A Publication of
the Society
for Industrial Microbiology

ANTIMICROBIAL AGENTS ANNUAL 1960

Proceedings of the
Conference
on Antimicrobial Agents
October 26-28, 1960,
Washington, D. C.,
L. G. Herman, Chairman

Sponsored by
the Society for
Industrial Microbiology

Distributed by PLENUM PRESS

$8.50 Domestic
$10.00 Foreign

ANTIMICROBIAL AGENTS ANNUAL 1960

Edited by
Peter Gray,
University of Pittsburgh

Proceedings of the Conference on Antimicrobial Agents, October 26-28, 1960, at Washington, D. C., sponsored by the Society for Industrial Microbiology, L. G. Herman, Chairman.

This volume is a compilation of over ninety of the papers presented at the 1960 Conference on Antimicrobial Agents, sponsored by the Society for Industrial Microbiology. These papers, which reflect the latest research in the field of antibiotics, cover the following general subject areas:

"New Products"

This section includes 18 papers presenting important new research data on such established antibiotics as penicillins, sulfonamides and peroxides, together with information on a number of recently developed antibiotic products which offer interesting possibilities for future use as therapeutic agents.

"Modes of Action"

Eleven papers under this general heading are among the first to be published on the subject of methods of inhibition by antibiotics. Effects on cell membrane permeability, protein synthesis, and cell wall synthesis, as well as competitive inhibition by various antibiotics are discussed.

continued on back flap

H. K. LEWIS & CO., LTD.
Booksellers,
136 Gower Street, London, W.C.1.

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ANTIMICROBIAL
AGENTS
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A Publication of the
Society for Industrial Microbiology

ANTIMICROBIAL AGENTS ANNUAL 1960

Proceedings of the Conference
on Antimicrobial Agents held in Washington, D. C.
October 26-28, 1960
under the chairmanship
of Lloyd G. Herman

Edited by

Peter Gray
Benjamin Tabenkin
S. G. Bradley

Distributed by
PLENUM PRESS, NEW YORK
1961

Library of Congress Catalog Card Number 61-15169

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Society for Industrial Microbiology
2000 P Street N. W.,
Washington 6, D. C.

Printed in the United States of America

INTRODUCTION

On October 26, 27, and 28, 1960, a Conference on Antimicrobial Agents was held in Washington, D. C. This conference was authorized by the Executive Board of the Society for Industrial Microbiology, at that time consisting of the following persons:

A. M. Kaplan, Ph.D., Quartermaster Research Center, Natick, Mass., President
F. Kavanaugh, Ph.D., Eli Lilly and Co., Indianapolis, Ind., Vice President
B. M. Miller, Ph.D., Merck Sharp and Dohme, Rahway, N. J., Secretary
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C. L. Porter, Ph.D., West Lafayette, Indiana, Director 1960-1962
L. S. McClung, Ph.D., Indiana University, Bloomington, Ind., Director 1960-1963
C. C. Yeager, Ph.D., Scientific Oil Compounding Co., Chicago, Ill., Past President,

and was thus officially sponsored by the Society for Industrial Microbiology. This board appointed the:

Committee

Conference on Antimicrobial Agents

* L. G. Herman, Ph.D., National Institutes of Health, Bethesda, Md., Chairman and Abstract Editor
* E. L. Dulaney, Ph.D., Merck Sharp and Dohme Research Laboratories, Rahway, N. J., Vice Chairman
* J. M. McGuire, Ph.D., Eli Lilly Research Laboratories, Indianapolis, Ind.
* W. M. Bejuki, D.Sc., National Academy of Sciences, National Research Council, Washington, D. C.
M. J. Pelczar, Jr., Ph.D., University of Maryland, College Park, Md.
P. K. Smith, Ph.D., George Washington University, Washington, D. C.
E. S. Feldmann, Ph.D., Scientific Division, American Pharmaceutical Association, Washington, D. C.
M. L. Robbins, Ph.D., George Washington University, Washington, D. C.
M. J. Romansky, M.D., George Washington University Hospital, Washington, D. C.

* Members SIM

Dr. L. G. Herman, as chairman of this board and abstract editor, bore the main responsibility for the preparation of the conference and for screening the contributions from the scientific viewpoint. He and his committee shouldered the burden of, and bear the responsibility for, collecting the papers in the pages that follow. The scholarly presentations, the academic merit, the facts, and the figures are the result of their efforts and to them must go the credit which is their due. There still, however, remained some tedious chores to be done. To do them, the Executive Board appointed an Editorial Committee, consisting of the following:

Editorial Committee for the Proceedings
of the Conference on Antimicrobial Agents

Peter Gray, Ph.D., Head, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pa.

Benjamin Tabenkin, M.S., Research Department, Hoffman-LaRoche, Inc., Nutley, N. J.

S.G. Bradley, Ph.D., Associate Professor of Bacteriology, University of Minnesota, Minneapolis, Minnesota

To this group has fallen the task of arranging the papers in logical sequence, of enforcing a minimum uniformity of headings and subheadings, of seeing that the style of one bibliography does not clash with the style of another, and those thousand and one other editorial duties which irk authors, but which, in point of fact, make life easier for readers. In this, they have been immensely helped by Mr. John Matzka, of Plenum Press, without whose continued and enthusiastic help this volume would never have appeared.

Peter Gray

The discussions which took place at the conference were recorded on tape to facilitate their inclusion in this volume. Unfortunately, the transcriptions were not always intelligible and occasional gaps do occur. However, the sense of the discussion is rarely obscured. The names of the speakers are given in most cases, but when the identity of the speaker could not be definitely established, only the letters Q and A were used to designate questions and answers.

When a group of papers covering the same general topic was presented, the discussion was delayed until the last paper in the group had been heard. In the book, in such cases, the discussion follows the last paper in the group.

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OPENING ADDRESS

Lloyd G. Herman

Chairman

Welcome to the first annual Conference on Antimicrobial Agents. We of the committee feel like calling this a "do-it-yourself" meeting because so many people have done so much in such a short time! In a 10-week period we have had the privilege of examining 101 abstracts—how many more would have been submitted had there been 10 months' notice we can only guess. Apparently "Antimicrobial Agents" are here to stay! Although the Society for Industrial Microbiology is but 10 years old, the intentional or accidental use of microbes for fermentation and oxidation reactions is as old as recorded history, e.g., yeast for bread and wine, and lactic acid bacteria in milk for cheese curds. Now within the past 20 years a third great era has dawned, namely, that of the conversion reaction, in which bacteria, molds, or streptomyces in enormous masses are utilized to convert air, water, and agricultural by-products such as whey, corn steep, stick water, sugars, and soy bean meal into medicinal and pharmaceutical products unknown 15 years ago.

In fact the "wonder drugs" have become household words in a truly international scale and the chief topic of conversation when two or three hospital survivors meet is "and what was your bug sensitive to?"

Penicillin is still very popular among research workers, who have now produced more than 40 varieties of the antibiotic—the latest being a synthetic product which will be described this morning. Serious studies are also continuing with the tetracyclines, the multitude of mycins, and the chemical agents such as the sulfonamides and the nitrofurans to mention but a few.

In the next few days we will hear much about several new compounds with new names—porfiromycin, tylosin, mikamycin, and geminimycin—showing that the microbiologist and etymologist are still working and producing.

We tend to agree with Prince Philip who was recently quoted as saying, "I am inclined to believe that no scientific organization is doing its job properly unless it takes a very active interest in the application of its findings. It is very tempting to turn out results and expect someone else to do something about them." The fact that this conference is being sponsored by the SIM indicates that we are interested in the ultimate use of our findings. The products of this new era of microbiology are useful not only as life-saving drugs or beneficial in crop and livestock production, but are also helpful in the preservation of many items of perishable food—if we can ever decide whether the antimicrobial agents we use or the bacteria we are trying to inhibit are the more dangerous to human health and welfare. Still undeveloped, but just as important scientifically, if not yet economically, are the many other products of microbial synthesis. I refer to the production of amino acids, growth factors, vitamins, or other stimulatory (not inhibitory) substances that can help plants and animals to resist the invasive characteristics of many of the presently known pathogenic species of microorganisms. Blood plasma extenders can be made from the microbial alteration of sucrose, vitamin B_{12} can be isolated from sewage, and steroid compounds are modified with the help of mold mycelium. Observe too, the natural everyday phenomenon by which some millions of ruminants the world over are being fattened on cellulose-digesting rumen bacteria. The list can be extended almost endlessly. For example, I have on my desk at the moment a letter from a general practitioner in which he states that he has evidence to show that a strain of Pseudomonas aeruginosa isolated from a cancerous tissue appeared to be responsible for the apparent cure of his patient. Before you smile or shake your head, don't forget that each one of us here, at least those of us with a few gray hairs, had an equal opportunity with Dr. Fleming to discover a use for the glistening yellow droplets so often noted on the surface of Penicillium mold cultures, the antimicrobial agent that is now providing us with so many strains of penicillin-resistant and troublesome staphylococci.

To come back to our original purpose, the committee authorized to conduct this conference is able to present 83 titles to you during the next three days, as well as three panels and a series of round table discussions. The sessions are divided roughly into 20 titles each in clinical uses, laboratory studies, and new products, 10 titles each in antifungal compounds, mode of action, and agricultural and nonmedical uses, and 3 on antitumor agents. We are sorry that the program will have to be divided into two concurrent sessions but we are extending the discussion period to at least five minutes for each paper. The questions and answers are being tape-recorded and will be transcribed for inclusion in the book to be published.

The aim and goal of the Society for Industrial Microbiology is primarily one of disseminating knowledge and information in free and open discussion through presentation of the results of studies of various groups of scientific workers such as chemists, physiologists, microbiologists, and clinicians, to name but a few. In this way the Society hopes to foster a "united front" for the problems that can be caused by or corrected by the effects of microbiological activities.

The newly isolated compounds produced by our microbial friends should not just be purified, numbered, and stored on the shelf, or their possible uses merely recorded as an idea in a conception book or treated as a secret and jealously guarded by the company librarian and the patent lawyer, nor on the other hand should they be released before they are thoroughly studied. To be of ultimate use, these products and concepts must be shared and used in studies covering many fields so that failure for one purpose does not automatically mean rejection in other categories. These conferences on antimicrobial agents, we hope, will quickly develop many facts; their recombination will provide the observant with some new information and eventually some knowledge will be confirmed and verified in other areas—but the wisdom involved in the proper use of antimicrobial agents will emerge much more slowly. In fact, by the year 2000, assuming no error in judgment has occurred, our 1960 Conference will have been reduced to a few feet of microfilm, to make room for more reports of the activities of other conferences in which we hope there will still be the opportunity to discuss freely and openly any theories or subjects proposed by individuals with the courage and ability to develop and present them.

We are optimistic in our hopes for the future. We do not want to limit the conference to SIM members nor do we want to exclude them. We feel that changes in chairman, committee members, and conference location are desirable; no one should be allowed to succeed himself more than once. Emphasis on different aspects of antimicrobial agents could be developed on a three to five year rotating basis, so that long-range studies could be encouraged—since results on hundreds of cases rather than on a few dozen are often more reliable. There is fortunately plenty of material available for separate three-day conferences on many aspects of antimicrobial agents. Let us not narrow our views, but rather lay out a broad highway so that the traveler seeking out metabolic pathways will not remain hidden in the woods, but will be able to move along at a respectable speed in whatever vehicle he chooses to travel.

Let me acknowledge the tremendous assistance I have had from all the members of the Committee. Walter Bejuki, Mary Louise Robbins, Mike Pelczar, and Ed Feldmann have been most helpful and encouraging when the going was tough. Gene Dulaney and Jim McGuire, at a distance, have stood by us through thick and thin. Monroe Romansky has been most valuable in convincing his colleagues that the Conference can and will include clinicians and has given freely from his busy schedule. It is with sincere regret that we have to hold the conference without the presence of Dr. Paul K. Smith, an outstanding pharmacologist from George Washington University. Dr. Smith died of a heart attack on October 6th. Prior to his death he reviewed all of the abstracts for the new products sessions and assembled them in the order in which they will be presented. Dr. David Gottlieb and Dr. John Ehrlich will preside over these sessions.

The chairmen of the sessions outlined in the program have willingly accepted these onerous chores and with the cooperation of the speakers and audience will do their best to guarantee "free speech" and discussion. Dr. Waksman, our honorary chairman, will address the group in this room at 1:30 and will describe his experiences on a recent European journey.

To Dr. Hiden Cox and Dr. John Olive and the staff of the American Institute for Biological Sciences, for their hospitality in permitting us unlimited use of their facilities, for their help and advice in organizing our program and press campaign, and especially to Miss Ann Barker, our secretary, clerk, and typist, in short our girl Friday, who kept all of us in check, we are indeed most grateful.

Let us now turn to the serious part of the Conference. May I introduce Dr. Romansky.

ANTIBIOTICS TODAY

A Survey of Accomplishments, Problems, and Potentialities

Selman A. Waksman
Professor Emeritus
Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey

With the discovery, in 1939-1940, of the first three important practical antibiotics, namely tyrothricin, a product of bacteria, penicillin, a rediscovery of a product of fungi, and actinomycin, a product of actinomycetes, it became evident that some central body would soon be required to coordinate the results obtained on the production, standardization, and utilization of these antibiotics. Unfortunately, the world was at that time in the midst of a great war. Each country was left to its own resources to do what it could in this new field that offered so much for the treatment of infectious disease. This was first recognized in the United States of America and in Great Britain, where the first three antibiotics were isolated. The Committee on Medical Research of the Office of Scientific Research and Development of the United States, headed by Dr. A.N. Richards, gave immediate consideration to the need for collaboration between different university, government, and industrial laboratories. A committee was appointed in 1940-1941 for the purpose of coordinating research and manufacture, as well as the standardization and evaluation, of penicillin and other antibiotics.

At first this Committee occupied itself primarily with problems pertaining to penicillin, since this appeared to be the most effective and least toxic antibiotic. With the end of World War II and with the establishment of standard requirements for antibiotics by the United States Food and Drug Administration, the above Committee ceased to function, its activities being taken over by other agencies of the U.S. Government such as the Keefer Committee for streptomycin. Attention must be called to the fact that the first committee was to a certain degree international in scope, since it worked in close collaboration with a comparable committee in Great Britain, at least as far as penicillin was concerned.

Streptomycin was the next important antibiotic that received universal attention. Although it was isolated and developed during the war years (1943-1945), both the antibiotic itself and the cultures producing this antibiotic were made available to all interested research and manufacturing organizations throughout the world. All the available information, including samples of the crude antibiotic material and the best available cultures, were widely distributed from the laboratories of the university where this antibiotic was first isolated.

As soon as the numerous industrial organizations became interested in the screening of antibiotic-producing organisms and as soon as new antibiotics were being isolated in numerous laboratories throughout the world, the situation underwent a complete change. This was true especially for those antibiotics that have found practical application as chemotherapeutic agents, such as chloramphenicol, chlortetracycline, oxytetracycline, and numerous others.

The extensive use of antibiotics, which soon came to be known in the popular mind as "miracle drugs," began to bring many problems with them. Certain severe reactions, due either to sensitization or to particular allergies of some individuals, were to be expected. Much more general and therefore more important was the development of resistance to the older and more extensively used antibiotics, notably penicillin and streptomycin. To meet these limitations, two approaches were developed, one consisting in the combination of certain antibiotics with other antibiotics or with synthetic compounds, and the other comprising the isolation of new antibiotics to which the resistant organisms are sensitive. Thus came into practical use, under a variety of different names, combinations of penicillin with streptomycin, of streptomycin with dihydrostreptomycin or with PAS or INH, and a variety of others. Numerous new antibiotics were isolated. An attempt to overcome the growing resistance of the tuberculosis organism to streptomycin led to the isolation of neomycin, viomycin, cycloserine, and others. Penicillin resistance led to the isolation of erythromycin, novobiocin, oleandomycin, vancomycin, and many others.

Other groups of disease-producing microbes not sensitive or insufficiently sensitive to the early antibiotics were not overlooked. Bacitracin, a bacterial product, was found to be primarily active against Gram-positive bacteria; polymyxin, also a bacterial product, was active largely against Gram-negative bacteria; nystatin, an actinomycete product, and griseofulvin, a fungal product, were both active upon disease-producing fungi. A variety of other antibiotics active upon bacteria, fungi, and protozoa soon came into the field to occupy important places in human and animal therapy. The effect of antibiotics upon various plant diseases was studied and effective results obtained, as illustrated by the use of cycloheximide and streptomycin. Animal feeding and food preservation have been benefited by the use of bacitracin, the tetracyclines, and some of the older antibiotics, notably penicillin and streptomycin.

Within 18 years after the successful introduction of the first three effective antibiotics, some 30 compounds have come into practical use as important chemotherapeutic agents. A new field of science and application was opened up. Diseases that only a generation ago were thought to be incurable became subject to successful therapy. This was true of various diseases of childhood, as well as of pneumonia, dysentery, typhoid and typhus fevers, plague and cholera, tuberculosis, and a variety of other infectious diseases caused by bacteria, fungi, and protozoa.

Unfortunately, this cannot be said of the true virus diseases and of various organic diseases. Although some definite progress appears to have been established in the treatment of some of these diseases, notably cancer, the tremendous efforts made in this direction have so far not been very rewarding.

CHEMICAL STRUCTURE vs BIOLOGICAL ACTIVITY

The following questions pertaining to the antimicrobial activities of antibiotics logically present themselves:

Why do some antibiotics act upon bacteria alone, others on fungi alone, and still others upon both bacteria and fungi ?

Why do some affect rickettsiae and the psittacosis-lymphogranuloma group of intracellular parasites, but not the true viruses ?

Why are the Gram-positive bacteria far more sensitive to the great majority of antibiotics produced by actinomycetes and other microorganisms than are the Gram-negative bacteria ?

Why do some antibiotics have a marked activity upon acid-fast bacteria and not others, even those that are active upon various Gram-positive organisms ?

Why do some bacteria and not others develop rapid resistance to some of the antibiotics, as in the case of staphylococci vs. streptococci to penicillin ?

Why do the rates of development of resistance differ for different antibiotics, as in the mechanism of development of resistance among sensitive bacteria to penicillin vs. streptomycin ?

Why do some bacteria produce strains that become nutritionally dependent upon certain antibiotics, as in the case of streptomycin ?

WHAT DOES THE FUTURE HOLD ?

It is believed in some quarters that the development of resistance to antibiotics suggests their reduced usefulness and gradual elimination. As new antibiotics are introduced, these as well are later followed by the development of resistance to them. The gloomy prophet tends to see in this the end of the antibiotic era. The optimist, however, is greatly heartened by the progress already made. He foresees in the near future the complete elimination of tuberculosis as the great enemy of man; he looks forward to the complete control of children's diseases. Such infections as undulant fever, typhoid, dysentery, cholera, plague, and venereal diseases, no longer hold for him the threat that he faced prior to the advent of the antibiotics. He even looks forward to a successful attack upon virus diseases and cancer. How soon this may come about only the future can tell.

You will thus recognize that we are now facing some very serious problems. Not the least important of them are those of organization. I should like to dwell upon only two important problems, one international and one national.

I have just attended, in Geneva, Switzerland, at the request of the World Health Organization, a meeting of a small committee on antibiotics. This is the third meeting of such a committee I have attended since 1950. It has now been decided to establish an international center for antibiotics and antibiotic-producing organisms. The Director-General of the WHO is now in the process of selecting a suitable geographic location for such a center.

The national problem that we are facing today is somewhat more difficult in character. The first symposium on antibiotics was held in 1946 in New York, under the auspices of the New York Academy of Sciences. Although this symposium was a great success, no attempt was made at that time to continue it on an annual basis or to establish an association of all those interested in the field to provide for suitable collaboration, presentation, and publication. Dr. Henry Welch, who was in an ideal position to recognize the importance of such annual symposia and who organized the first regular symposium (after an irregular previous one) on October 28, 1953, said in his introduction: "This symposium should help greatly to orient the scientific worker on the present status of antibiotic therapy and development. Ten years ago penicillin was the only antibiotic known to medical practice, and its use was confined to battlefield casualties. There was none available for civilian patients. Today there are numerous antibiotics, accounting for more than one-half of all prescriptions written in this country. The past ten years may well go down in medical history as an 'Antibiotic Era,' based upon what these drugs have done to minimize human suffering and extend the life span."

Dr. Keefer, in his message of welcome, said: "It is impossible to completely assess the benefits that have flowed from the discovery and development of antibiotics, but no one will deny that they have been a blessing to mankind."

It appears that now we have reached an impasse. However, just as the proverbial phoenix, we may now have the great opportunity for which we have been waiting for 20 years. It is being proposed that an association be organized, to hold meetings, publish one or more journals, and provide for the proper dissemination of information concerning a subject which is of importance to us all, the subject of antibiotics.

ENZYMATIC CLEAVAGE AND RESYNTHESIS OF PENICILLINS

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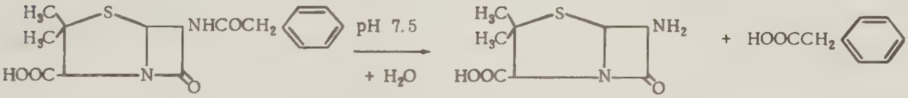
The production of 6-aminopenicillanic acid and its isolation from submerged cultures of *Penicillium chrysogenum* have been described by Batchelor et al. [1]. An enzymatic hydrolysis of the side chain of benzylpenicillin has been reported by Sakaguchi and Murao [2] in 1950, but has, as far as we know, not been confirmed by other workers.

Early in 1959 we succeeded in enzymatically hydrolyzing penicillin G to 6-aminopenicillanic acid (6-APA) and phenylacetic acid, using washed cells of *Escherichia coli* and other bacteria. This reaction (Reaction 1) proceeds well in phosphate buffer solutions at pH 7.5 or slightly more alkaline pH values at a temperature between 20 and 40 C in the presence of toluene, but requires frequent additions of sodium carbonate to keep the pH constant. The reaction requires 3 to 6 hr to approach quantitative completion. Since the hydrolyzing enzyme is integrated rather firmly in the cell-wall—membrane complex of *E. coli*, the bacterial cells separated from the reaction mixture may be re-used for further runs. By conventional methods the reaction mixture, freed from bacterial cells, is acidified and extracted for the removal of phenylacetic acid and some residual penicillin G. After adjustment of the separated aqueous phase to pH 4.3, 6-APA crystallizes spontaneously in a good yield.

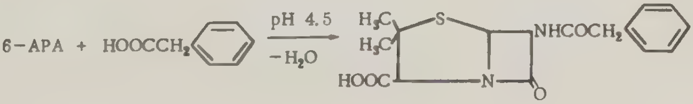
Reaction 1:



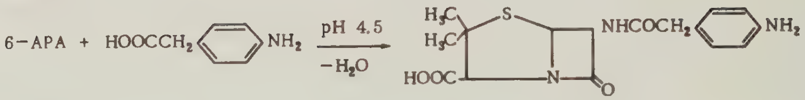
benzylpenicillin — 6-aminopenicillanic acid = 6-APA — + phenylacetic acid

The enzymatic reaction cleaving the acyl side chain of benzylpenicillin in neutral to slightly alkaline media proved to be reversible. It proceeds in the opposite direction at acid pH values. At pH 4.0 to 6.0 in the presence of 6-APA and phenylacetic acid, cells of *E. coli* are able to synthesize benzylpenicillin (Reaction 2). Substituting a series of other carboxylic acids, including aliphatic acids, for phenylacetic acid, it is possible to yield the corresponding penicillins, though at slow reaction rates. For example, Reactions 3 to 5 lead to the synthesis of p-aminobenzylpenicillin, n-pentylpenicillin, and ethylmercaptomethyl-

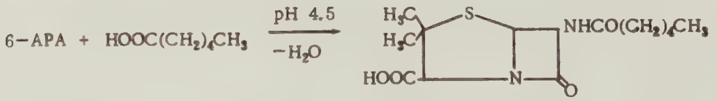
Reaction 2:



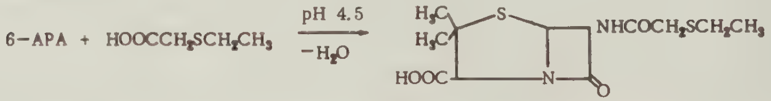
Reaction 3:



Reaction 4:

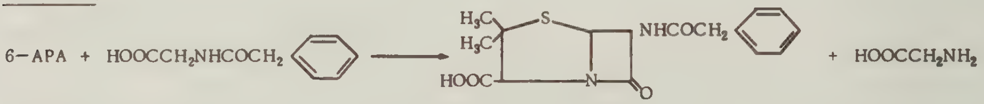


Reaction 5:

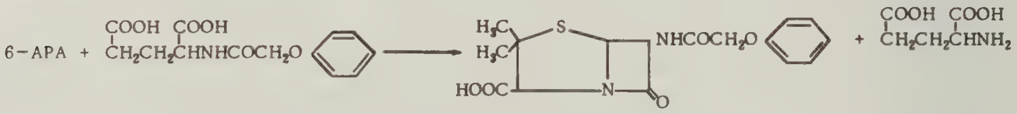


penicillin. By application of the relatively energy-rich phenylacetyl glycine instead of free phenylacetic acid or its salts, benzylpenicillin is formed in a very fast reaction, phenylacetic acid being transferred thereby from glycine to 6-APA (Reaction 6). For this type of reaction other acylamino acids may be employed, diverse in the acyl residues as well as in the amino acids: 6-APA + phenoxyacetyl glutamic acid → phenoxymethylpenicillin + glutamic acid (Reaction 7); 6-APA + o-chlorophenylacetyl analine → o-chlorobenzylpenicillin + alanine (Reaction 8).

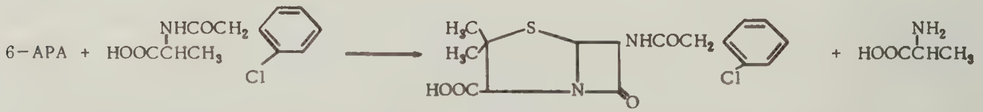
Reaction 6:



Reaction 7:

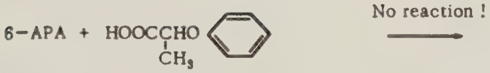


Reaction 8:

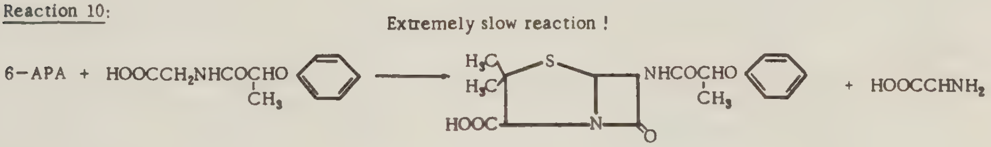


The synthesis of penicillins by reaction of 6-APA and acylamino acids derived from disubstituted acetic acids is, however, extremely slow. Thus the yield of α-phenoxyethylpenicillin resulting from the enzymatic reaction between α-methylphenoxyacetyl glycine and 6-APA is very low (Reaction 10), and 6-APA does not react at all with free α-methylphenoxyacetic acid (Reaction 9). In contrast, we obtained high yields of α-phenoxyethylpenicillin by the action of E. coli on a mixture of α-methylphenoxyacetyl thioglycollic acid and 6-APA, thioglycollic acid being split off in this reaction (Reaction 11). Since the reaction proceeds both in acid and in slightly alkaline media, it is thought to be irreversible. Hence, at neutral to alkaline pH the bacterial enzyme is able to effect a rapid and nearly quantitative conversion of benzylpenicillin to α-phenoxyethylpenicillin

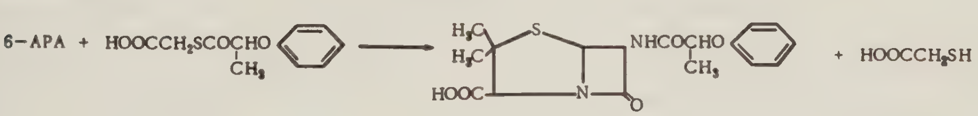
Reaction 9:


6-APA + HOOCCHO
CH3
No reaction !

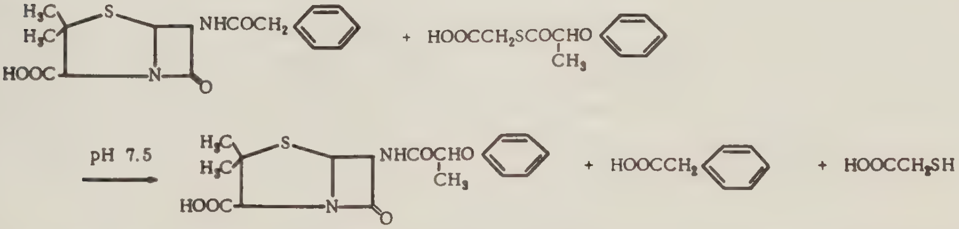
Reaction 10:


Extremely slow reaction !
6-APA + HOOCCH2NHCOCHO
CH3
H3C
H3C
S
HOOC
N
O
NHCOCHO
CH3
+ HOOCCHNH2

Reaction 11:


Fast reaction !
6-APA + HOOCCH2SCOCHO
CH3
H3C
H3C
S
HOOC
N
O
NHCOCHO
CH3
+ HOOCCH2SH

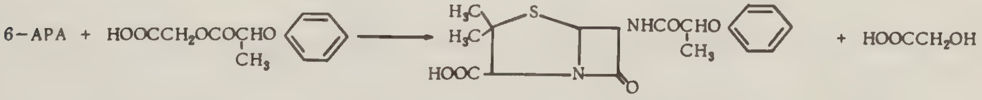
Reaction 12:


H3C
H3C
S
HOOC
N
O
NHCOCH2
+ HOOCCH2SCOCHO
CH3
pH 7.5
NHCOCHO
CH3
+ HOOCCH2
+ HOOCCH2SH

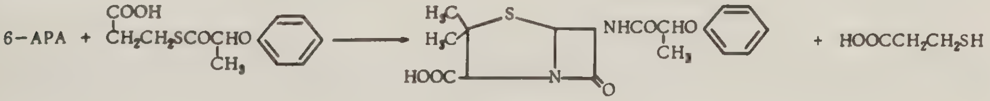
in the presence of α-methylphenoxyacetyl thioglycollic acid (Reaction 12). Similarly, other relatively energy-rich compounds may be used for the transfer of acyl residues to 6-APA. These are acylated hydroxycarboxylic acids, mercaptocarboxylic acids, phenols, and thiophenols, as is shown in Reactions 13 to 16: 6-APA + α-methylphenoxyacetyl glycollic acid → α-phenoxyethylpenicillin + glycollic acid; 6-APA + α-methylphenoxyacetyl-β-thiopropionic acid → α-phenoxyethylpenicillin + β-thiopropionic acid; 6-APA + α-methylphenoxyacetylphenylic ester → α-phenoxyethylpenicillin + phenol; 6-APA + α-methylphenoxyacetylthiophenylic ester → α-phenoxyethylpenicillin + thiophenol. In the absence of the enzyme a synthesis of penicillins in mixtures of the above-mentioned acylated compounds and 6-APA could not be detected.

The enzyme of *E. coli*, the biochemical activities of which were described above, is rapidly and irreversibly inactivated in acid media below pH 4.0. Its

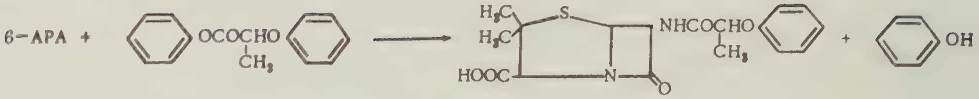
Reaction 13:


6-APA + HOOCCH2OCOCHO
CH3
H3C
H3C
S
HOOC
N
O
NHCOCHO
CH3
+ HOOCCH2OH

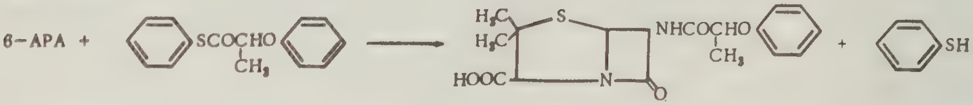
Reaction 14:


COOH
6-APA + CH2CH2SCOCHO
CH3
H3C
H3C
S
HOOC
N
O
NHCOCHO
CH3
+ HOOCCH2CH2SH

Reaction 15:



Reaction 16:



activity seems to be independent of the presence of any cofactors. Considering its location at the surface of the bacterial cells, the normal biochemical function of the enzyme may be involved in the transport of certain "nutrients" and metabolites across the cell wall or the cell membrane into either the cell or the surrounding culture medium. According to its biochemical activities we tend to classify the enzyme as an acyl transferase.

SUMMARY

Reversible enzymatic cleavage of benzylpenicillin to 6-aminopenicillanic acid (6-APA) and phenylacetic acid by cells of Escherichia coli is reported. The enzyme responsible for this reaction represents an acyl transferase that is able to synthesize penicillins by transferring acyl residues from acylamino acids, acylated hydroxycarboxylic acids, mercaptocarboxylic acids, phenols, and thiophenols to 6-aminopenicillanic acid.

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DISCUSSION

Q: Did your E. coli strain also produce a penicillinase?

A: Very small quantities of penicillinase.

Q: You didn't find this sufficient to cause difficulties in your reaction?

A: We are able to separate this penicillinase from the cells. This penicillinase is a slight nest-egg constituent of the cells.

Q: I wonder what concentrations you are using in your substrates?

A: We use concentrations of about 3 to 6% penicillin G for testing.

Q: I'm curious, what criterion did you use for knowing that you had only cell wall which carried out this reaction?

A: Addition of some toluene to suspensions of E. coli makes the cell wall and the cell membrane permeable for cytoplasmic constituents. As a result, nucleic acids and proteins, especially free penicillinase, can be detected in the supernatants of centrifuged bacterial suspensions. The enzymatic activity hydrolyzing penicillin is recovered from the sediment.

Q: Let me ask you again, when you made your cell wall separation you said that cell wall fractions may contain some of the cell membrane that is also active. The cell wall is active. Is that right? Now, the question was asked, How did you know that this was cell wall or cell membrane and was not contaminated with the other fractions? Did you centrifuge it several times? What did you do? Did you examine it under a microscope? How did you know it was relatively pure?

A: Well, after contact with toluene we washed our cells by centrifuging and resuspending them several times. Microscopically the recovered cell envelopes looked rather empty and they could be stained only badly with basic dyes, as is the case when cytoplasmic contents are leached out of the cells. We could see that it is possible to elute the largest part of the hydrolyzing enzyme from these "empty" cell envelopes by addition of bile salts or other surface-active agents that don't destroy the enzyme. By this procedure at least some material of the cell membrane is solubilized.

Q: Can you increase your enzymatic activity by culturing your E. coli in the presence of some penicillin?

A: No, it is not possible to increase the activity by adding some penicillin to the culture, but by certain other procedures it is possible to yield more enzymatic activity. I am sorry not to be able to say more about this at the moment.

MICROBIOLOGICAL PROPERTIES OF A NEW SYNTHETIC PENICILLIN, 2,6-DIMETHOXYPHENYLPENICILLIN*

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Research Division, Bristol Laboratories, Syracuse, New York

INTRODUCTION

The discovery of methods of making 6-aminopenicillanic acid in quantity [1, 2, 3] made possible the synthesis and evaluation of a great variety of penicillins. For the first time, various properties of penicillin could be related to specific configurations of the side chain. Thus, the properties of one series of synthetic penicillins having as a side chain α-aryloxyalkyl have been reported [4].

Recently, a new synthetic penicillin, 2,6-dimethoxyphenylpenicillin, has been described. The reports showed that this penicillin is extremely resistant to the action of Staphylococcus aureus and Bacillus cereus penicillinases [5, 6, 7]. It is, however, an efficient penicillinase inducer. Systems in which a compound is an enzyme inducer but not an enzyme substrate are well known, the best studied among them being the β-galactosidase induction by melibiose investigated by the Monod group [8].

2,6-Dimethoxyphenylpenicillin was shown to be endowed with several attributes of a true penicillin. Among them are its antibacterial spectrum, its bactericidal (as distinguished from bacteriostatic) action, and its activity against growing cells and lack of activity against resting cells. On the other hand, the over-all activity of this compound on a weight basis was shown to be appreciably lower than that of other penicillins.

The present work was directed toward the investigation of other properties of this penicillin. Studies were carried out attempting to determine if 2,6-dimethoxyphenylpenicillin has a mechanism of action similar to that of natural penicillin. It has been shown that penicillin acts by interfering with cell wall synthesis [9]. One of the consequences is that cells show an increased osmotic fragility. In ordinary media, such cells will lyse. By using hypertonic media, such cells are protected and take on forms known as "protoplasts" or "spheroplasts" [10]. The present study concerns itself mostly with the comparison of the lesion produced by 2,6-dimethoxyphenylpenicillin and that produced by benzylpenicillin (penicillin G).

MATERIALS AND METHODS

Escherichia coli K-12 and B. cereus ATCC 9634 used in this study were maintained on nutrient agar slants.

Spheroplast formation by E. coli was observed by following the Lederberg technique [10]. Osmotic fragility of the same organism was studied by the method described by Hurwitz et al. [11], except that the turbidity was measured in a Beckman B spectrophotometer at 650 mμ instead of with a nephelometer.

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*The trademark of Bristol Laboratories, a division of Bristol-Myers Company, for 2,6-dimethoxyphenyl penicillin is Staphcillin.

Spheroplasts from B. cereus were produced by a modification of the method described by Foldes and Meretey [12]. Best results were obtained by the following procedure: Twenty milliliters of antibiotic assay broth (Baltimore Biological Laboratory) was inoculated from a slant culture and incubated overnight at 30 C. In the morning, 80 ml more of antibiotic assay broth was added and the culture was incubated for an additional 4 hr. This was done to ensure that the culture was in the logarithmic growth phase, a very important point as described by Foldes and Meretey and confirmed in our laboratories. The culture was then centrifuged, and the concentrated cells were resuspended in 5% of the original volume. Then, 0.5 ml of this suspension was added to a broth consisting of 10 ml of antibiotic assay broth, 1.5 ml of 0.1 M solution of $MgSO_4$, and 5 ml of 2.4 M sucrose solution. To each of four such preparations, benzylpenicillin was added to make 10 and 100 μg/ml solutions and 2,6-dimethoxyphenylpenicillin to make 100 and 1000 μg/ml solutions. The treated cultures were incubated at 30 C for 15 min on the shaker, then in still culture. Visual observations were made from time to time with a phase-contrast microscope.

RESULTS

Experiments with E. coli failed to show the appearance of true spheroplasts when the culture was incubated with different concentrations of 2,6-dimethoxyphenylpenicillin, although controls incubated with benzylpenicillin showed a high proportion of spheroplasts. Close observation revealed that 2,6-dimethoxyphenylpenicillin caused swelling and increase in size of the individual cells in a fashion similar to the first stages of spheroplast formation with benzylpenicillin, but these enlarged and swollen cells did not evolve into spheroplasts.

Osmotic fragility is one of the consequences of penicillin action. Spheroplasts, formed by incubating cells in a nutrient medium containing benzylpenicillin and a high concentration of sucrose, lyse when the medium is diluted to reduce the sucrose concentration. For this organism, the protection by sucrose is quite specific. According to Hurwitz et al., the lysis can be best measured by the decrease in optical density of the diluted suspension caused by heating it for 4 min in a 56 C water bath. Figure 1 represents the drop of optical density in function of time when E. coli is treated with either 300 μg/ml benzylpenicillin or 20 mg/ml 2,6-dimethoxyphenylpenicillin. Both penicillins induce osmotic fragility, but with benzylpenicillin this occurs more rapidly, the peak of fragility being reached in 2 hr. This agrees well with visual observation of the formation of protoplasts, the maximum also being reached in 2 hr. The appearance of osmotic fragility with 2,6-dimethoxyphenylpenicillin occurs later than with benzylpenicillin. The high concentration of 2,6-dimethoxyphenylpenicillin was used in such an experiment because it is considerably less active than benzylpenicillin.

In order to determine if such a high concentration might interfere with spheroplast formation, E. coli was treated as above using both 20 mg/ml 2,6-dimethoxyphenylpenicillin and 300 μg/ml benzylpenicillin. Spheroplast formation occurred as readily as in the benzylpenicillin controls.

Foldes and Meretey described "protoplast" formation in B. cereus by benzylpenicillin. It was thought worthwhile to determine if such forms could be produced in a Gram-positive organism, where lower concentrations of the antibiotic could be used. It was found that formation of up to 50% protoplasts was obtained when a logarithmically grown culture of B. cereus was incubated with 1000 μg/ml 2,6-dimethoxyphenylpenicillin (hypertonic solution). Smaller percentages of protoplasts were formed under the influence of 100 μg/ml 2,6-dimethoxyphenylpenicillin and this antibiotic produced spheroplasts more readily than benzylpenicillin in these experiments. This is probably due to the fact that the penicillin is quite resistant to the penicillinase produced by B. cereus while benzylpenicillin is rapidly destroyed by it.

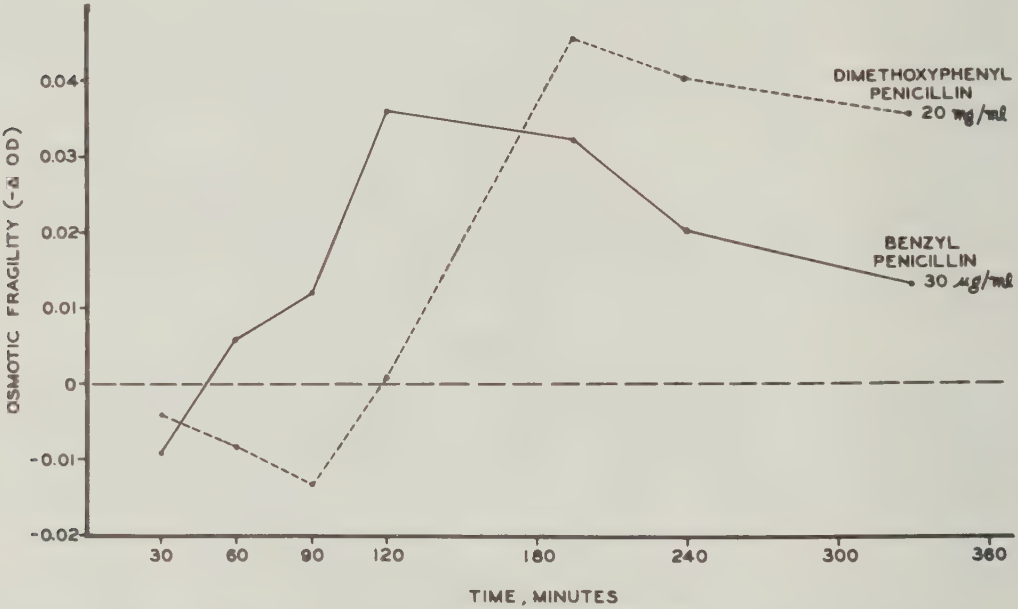

Fig. 1. Osmotic fragility measured as drop in optical density of diluted E. coli suspensions. The organism was grown in hypertonic sucrose medium containing 300 μg/ml benzylpenicillin or 20 mg/ml 2,6-dimethoxyphenylpenicillin. As indicated, time samples were diluted in distilled water and optical density determined before and after heating at 56 C.

DISCUSSION AND SUMMARY

The new synthetic penicillin, 2,6-dimethoxyphenylpenicillin, has many of the properties of natural penicillins. Its uniqueness resides in its extreme stability toward Staphylococcus aureus and Bacillus cereus penicillinases. While it has a spectrum similar to those of natural penicillins, it is considerably less active. The experiments described above indicate that despite the marked difference in activity, the new penicillin has the same mechanism of action as do the natural penicillins. The primary lesion appears to be in the cell wall, and cells affected by this penicillin become osmotically fragile. While no typical spheroplasts could be demonstrated with Escherichia coli, they could be readily produced using the more sensitive organism, B. cereus.

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DISCUSSION

Q: It's very interesting that you found that this compound is an inducer of penicillinase both of the type formed by Bacillus cereus and of the type formed by staphylococci. There is a rather confusing picture in the staphylococci, as we all know. There is that type of penicillinase formation which occurs in the test group on continued transfer, and there is that intracellular type which occurs in clinical cases. In studying induced penicillinase formation, what type of staphylococcus penicillinase is referred to here?

A: We used whole cells.

THE INCREASED ACTIVITY OF RISTOCETINS A AND B FOLLOWING ACID HYDROLYSIS

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INTRODUCTION

Ristocetins A and B are very closely related, high-molecular-weight (~4000), amphoteric antibiotics active against Gram-positive bacteria and mycobacteria. A crystalline commercial preparation* which is largely ristocetin A sulfate has been in use for over two years.

The isolation and chemical properties, the pharmacological and toxicological properties, and the microbiological and clinical studies were reported earlier [1-7].

This paper presents observations on some acid degradation products of ristocetins A and B with emphasis on the very substantial increases in in vitro and in vivo activity. These appear to be the first reported examples of the formation of antibiotic degradation products which are decidedly more active than and which do not share complete cross-resistance properties with the parent substances. Quantitative determinations of each of the four sugars contained in both ristocetins A and B are also briefly described.

IN VITRO OBSERVATIONS

In the course of studies involving the carbohydrate portions of these molecules, it was found that when either of the ristocetins was hydrolyzed under mild acid conditions, the sugars were gradually liberated and the Bacillus subtilis plate-diffusion assays decreased. The presence of active degradation products was first suggested when an additional, elongated, slower-moving zone of inhibition appeared in the paper chromatograms of the acid hydrolysates of ristocetin A (Fig. 1).

Although the Rf value of this new chromatographic zone was identical to that of ristocetin B, it was subsequently found that this slower-moving zone of inhibition was produced by a complex of hydrolysis products. These differed from one another and from ristocetin B in carbohydrate content, dilution and plate assays, and ultraviolet absorption. Similar paper chromatograms of mild acid hydrolysates of ristocetin B were also indistinguishable from those of ristocetin B. Thus, the acid degradation products of ristocetins A and B and ristocetin B itself had similar Rf values in this system. Other systems were tried but were unsatisfactory for differentiating between these degradation products and ristocetin B.

Despite an apparent loss of activity (as measured by the plate-diffusion assay), it was subsequently found that the activity, measured by the broth dilution technique, actually increased substantially during hydrolysis (Fig. 2). These increases in activity were substantiated against six other sensitive organisms.

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*The trade name of Abbott Laboratories for ristocetin is "Spontin."

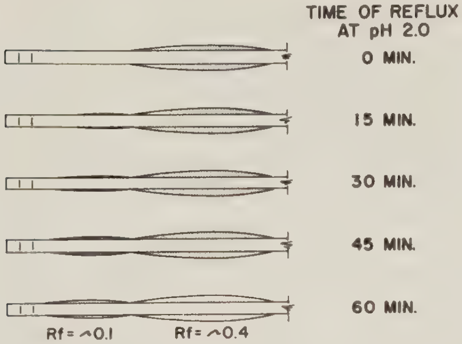

Fig. 1. Paper chromatograms on acid hydrolysates of ristocetin A.

Four- to thirtyfold increases in activity were observed (Table 1). Although ristocetin B is several times more active than ristocetin A, the most active degradation products of both were approximately equally active.

Surprisingly, no cross resistance between the acid degradation products of ristocetins A and B and the parent compounds could be demonstrated using *Staphylococcus aureus* 67AR, in which resistance to ristocetin A was developed in the laboratory [2].

This lack of cross resistance, however, was in one direction only. When culture *S. aureus* 67 was made resistant to a mildly hydrolyzed (approximately 10 min) ristocetin A, it was also found to be resistant to ristocetin A.

In this experiment five cultures were studied but only one, *S. aureus* 67, developed a significant degree of resistance to the hydrolyzed ristocetin A preparation. This resistance appeared rather abruptly at the thirteenth and fourteenth transfers. The culture at this point became very granular, grew more slowly,

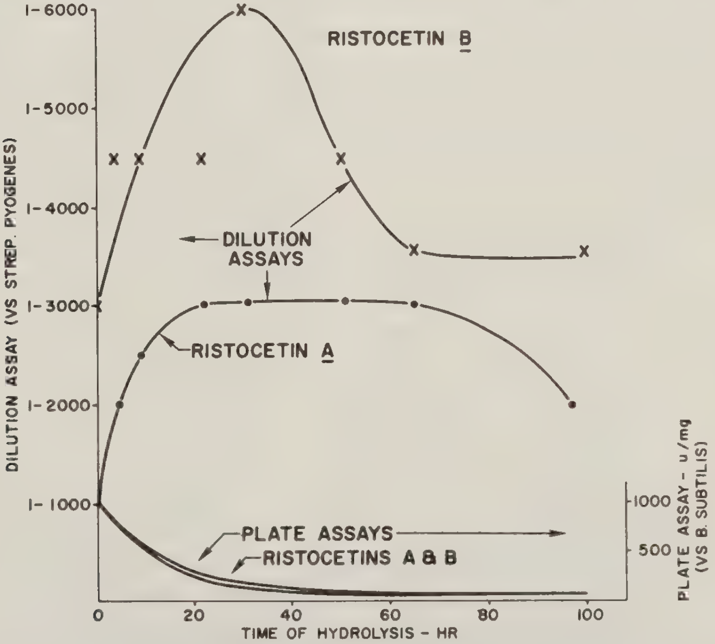

Fig. 2. Plate and dilution assays on acid hydrolysates of ristocetins A and B in boiling water at pH 2.0.

Table 1. In Vitro Activities of Hydrolyzed Fractions of Ristocetins A and B (48-hr inhibitory concentration, μg/ml)

Organism	Hydrolysis* of ristocetin A					Hydrolysis* of ristocetin B				
	0 min	10 min	25 min	50 min	75 min	0 min	10 min	25 min	50 min	75 min
S. aureus 67	25	1.6	0.8	1.2	1.6	3.1	0.8	1.6	0.8	0.8
S. aureus 67AR	>100	1.6	0.8	0.4	0.8	>100	0.8	0.8	0.6	0.8
S. albus 3519	19	3.1	1.6	1.6	1.2	3.1	1.6	0.8	0.8	1.6
Enterococcus 89	6.2	2.3	1.6	1.6	3.1	3.1	1.6	1.6	1.6	3.1
S. pyogenes C2O3	0.8	0.2	0.2	0.2	0.4	0.6	0.2	0.1	0.4	0.4
B. subtilis ATCC 10707	1.2	0.3	0.4	0.4	0.8	0.4	0.2	0.2	0.4	0.4
Corynebacterium species	0.8	0.2	0.2	0.4	0.6	0.3	0.1	0.2	0.4	0.6
Diplococcus pneumoniae 6301	3.1	0.8	0.8	1.6	1.6	0.8	0.8	0.8	1.6	1.6

*A 10% solution of each of the ristocetins in 1 N H_2SO_4 was refluxed for the times shown. The cooled solutions were neutralized with NaOH and the insoluble hydrolysis products were filtered and dried. Each of these isolated hydrolysis products was equivalent to approximately 60-70% by weight of the starting materials.

and was stimulated by the presence of ristocetin, suggesting that a mutational change may have taken place.

The degree of resistance development as judged by growth on serial transfer is shown in Table 2.

IN VIVO OBSERVATIONS

In order to determine whether the pronounced increase in activity observed in the broth dilution assay was also demonstrable in vivo, the hydrolysates of both ristocetin A and B were compared with the parent antibiotics in mice infected with staphylococci. The same 10- and 50-min hydrolysates used for the in vitro experiments (Table 2) were used for the mouse protection tests. Table 3 summarizes the data of mouse protection tests with ristocetins A and B and their hydrolysis products. The hydrolysates of ristocetin A were about five times as active as ristocetin A itself. Hydrolysates of ristocetin B were two to four times as active as the parent antibiotic. These increases in activity in the mouse protection test are thus of the same order of magnitude as the in vitro broth dilution assays.

Table 2. Development of Resistance to Hydrolyzed Ristocetin A

Culture	Number of transfers	Increase in resistance
S. aureus 45	25	1×
S. aureus 67	12	8×
S. aureus 67	14	64×
S. aureus 67	25	256×
S. albus 3519	25	2×
Enterococcus 89	25	2×
Enterococcus 93	25	1×

In the direct comparison, the above resistant strain of S. aureus required 150 μg/ml of hydrolyzed ristocetin A for inhibition and 400 μg/ml of ristocetin A.

SOME CHEMICAL OBSERVATIONS

In addition to the four sugars already described as being liberated by mild acid hydrolysis, two other spots appeared simultaneously with these sugars on paper chromatograms. One (SM) was slower moving and the other (FM) was faster moving than the four sugars in the ethyl acetate—acetic acid—H_2O system (44 : 20 : 10). When SM was eluted from the paper, hydrolyzed further, and reapplied to the paper chromatogram, the four sugars appeared in unequal amounts. Considerably more rhamnose and glucose were present than D-arabinose and mannose, suggesting that the four sugars may, at least partly, break off as an oligosaccharide.

FM is apparently the first amino fragment to be liberated from the ristocetins A and B. It begins to appear (by paper chromatograms) very early (4–6 min) in the 1 N acid hydrolysis and its liberation from the ristocetin molecules may have some relation to the increased activity and lack of cross-resistance properties. This substance gives a positive ninhydrin test, a positive reducing test with $AgNO_3$, and has no appreciable UV absorption. FM is not as stable in acid as amino acids in general and cannot be detected in preparations which have been hydrolyzed for a longer time with stronger acids. More vigorous hydrolysis of the ristocetins liberates other amino fragments, some of which are phenolic.

Complete inactivation of the ristocetins occurs in 2–3 hr when a 10% solution of either ristocetin is refluxed in 1 N H_2SO_4 (the same conditions which were used in Table 1). The four sugars (glucose, mannose, D-arabinose, and rhamnose) were completely liberated from the molecule in 2 hr, although a very active sugar-free fraction was obtained by chromatography from a preparation which had been refluxed only 35 min.

No qualitative or quantitative changes in UV absorption were noted in preparations which were refluxed in 1 N acid for 3 hr. The 280 mμ peak, which is apparently due to the phenolic constituents, remained unchanged.

Table 3. Effect of Acid Hydrolysis on Activity of Ristocetin A and B Against Staphylococcal Infection in Mice

Ristocetin	Time of hydrolysis, min	CD_{50}, mg/kg	CD_{50}, 95% confidence limits
A	0	2.3	1.8-2.9
	10	0.42	0.35-0.50
	50	0.45	0.36-0.56
B	0	1.0	0.74-1.35
	10	0.40	0.33-0.50
	50	0.25	0.17-0.35

Crystalline ristocetins A and B and the products from each after acid hydrolysis for either 10 or 50 min in refluxing 1 N H_2SO_4 were examined in mouse protection tests with S. aureus Smith. Mucin was used as adjuvant for the staphylococcal infection. CFI mice weighing 16-18 g were infected by the intraperitoneal route with 100 LD_{50} of the staphylococci. The antibiotics were administered intramuscularly in four equal doses at 1, 3, 5, and 7 hr after infection. Statistical calculations were done by the method of Litchfield and Wilcoxon [8] to give the 50% survival dose (CD_{50}) and the 95% confidence limits.

Chromatographic fractionations were carried out with H_2SO_4-washed alumina on a 35-min 1 N H_2SO_4 hydrolysate of ristocetin A (which was near the peak activity based on the data in Table 1). Elution of the hydrolysate (less the liberated carbohydrate portion) with a 50 : 50 methanol-water solution yielded three distinct fractions (Table 4), each of which was combined on the basis of a differential in the B. subtilis plate and Streptococcus dilution assays.

When fraction II was further hydrolyzed, no sugars could be detected by paper chromatography, but a substantial amount of the amino fragment FM appeared. Fractions I and III yielded small amounts of each of the four sugars and only about one-tenth as much of the amino fragment FM. Fraction II was the most active according to the more significant Streptococcus pyogenes dilution assay and also apparently had a smaller proportion of the phenolic constituents as suggested by the UV absorption.

Fractions I and III were very widely separated on the chromatogram. Fraction I had greater UV absorption and was more water-soluble in the neutral pH range. In general, the degradation products became less water-soluble as hydrolysis proceeded and as the sugars and other fragments were liberated.

Attempts were made to crystallize these and other chromatographically separated degradation fractions, but without success. Differential assays, paper chromatography of the hydrolyzed, liberated sugars, UV absorption, and bio-paper chromatograms suggest that these fractions are complexes.

Table 4. Properties of Ristocetins and Their Hydrolysis Products

Preparation	Plate assay, units/mg	Streptococcus dilution assay, μg to inhibit	$E_{1\%}$ at 280 mμ	Sugars, % by wt
Ristocetin A	1000	0.93	45	40
Ristocetin B	1000	0.31	49	30
35-min hydrolysate of ristocetin A	85	0.23	72	–
Fraction I	215	0.93	77	~5 or less
Fraction II	56	0.15	65	0
Fraction III	125	0.46	69	~5 or less

QUANTITATIVE DETERMINATIONS OF THE SUGARS

The identification of the sugars, glucose, mannose, D-arabinose, and rhamnose was accomplished on neutral, hydrolyzed preparations by the use of paper chromatography and observations on the nutritional requirements of specific microorganisms.

The sugars xylose, fucose, fructose, galactose, and ribose were each eliminated by comparisons with the unknowns in one or more of the following paper chromatographic systems: butyl acetate—pyridine—water (5 : 3 : 1), phenol saturated with water, benzyl alcohol—acetic acid—water (8 : 3 : 1), and butanol—pyridine—water (6 : 4 : 3). In each of these systems the four unknown sugars compared exactly in Rf values with standard glucose, mannose, D-arabinose, and rhamnose.

The spray reagents, alkaline silver nitrate [9], aniline hydrogen phthalate [10], and aniline phosphate [11] were also used to detect the spots on the paper chromatograms. The colors and the rate of appearance of these colors were identical for the knowns and unknowns.

Table 5. Sugar Content of Ristocetins

Sugar	Ristocetin A		Ristocetin B	
	mg/g	moles/mole*	mg/g	moles/mole*
Glucose	82	1.9	86	2.0
Mannose	164	3.8	84	1.9
D-Arabinose	74	2.1	36	1.0
Rhamnose	79	2.0	79	2.0

*These values are based on the assumption that the D-arabinose found in ristocetin B represents 1 mole.

Known mixtures of the four sugars were compared with the unknown mixtures by incubation with certain microorganisms. Both *Salmonella typhosa* and *Saccharomyces cerevisiae* utilized glucose and mannose but did not utilize the arabinose and rhamnose. *Salmonella paratyphi* utilized glucose, mannose, L-arabinose, and rhamnose but did not utilize D-arabinose. Thus the D-configuration for arabinose was established. The presence of glucose was also indicated by the use of glucose oxidase.

The quantitative determinations of the sugars of ristocetins A and B were accomplished by a modification of a method used by Saeman et al. [12], and consists of the following steps: (a) very mild H_2SO_4 hydrolysis to liberate the sugars, (b) neutralization with $Ba(OH)_2 \cdot 8H_2O$, (c) chromatographic separation using ethyl acetate—acetic acid—water (44 : 20 : 10), (d) elution of each of the sugars from the paper, and (e) spectrophotometric determination of the individual separated sugars with the improved sugar reagent of Somogyi [13] and the arsenomolybdate chromogenic reagent of Nelson [14].

The four standard sugar solutions could be determined to within 10% of the theoretical values by this method.

The quantitative values obtained for the individual sugars present in the ristocetins were determined after a 2 hr refluxing in 1 N H_2SO_4 and are given in Table 5.

These results indicate that ristocetins A and B contain equivalent amounts of glucose and rhamnose and that ristocetin A contains twice as much of D-arabinose and mannose. These data also suggest that the molecular weights of the ristocetins are approximately 4000, which is in fairly good agreement with the values reported earlier [3].

ACKNOWLEDGMENT

The authors wish to acknowledge the help of T. Oliver for paper chromatography, S. Bell for plate assays, C. Vojtko for tube dilution tests and resistance studies, E. Alford for identification of sugars by fermentation tests, and R. Bower for in vivo studies.

SUMMARY

When ristocetins A and B were hydrolyzed with acid, four sugars and an amino fragment were liberated from the molecules and the activities increased four- to thirtyfold against the sensitive Gram-positive organisms. These degradation products did not retain cross-resistance properties with the parent ristocetins A and B against a *Staphylococcus aureus* strain (67 AR) which was made resistant to ristocetin A. Increased *in vivo* activity was also demonstrated.

Ristocetin A was found to contain 4 moles of mannose and 2 moles each of glucose, D-arabinose, and rhamnose. Ristocetin B was found to contain 1 mole of D-arabinose and 2 moles each of mannose, glucose, and rhamnose.

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PORFIROMYCIN, A NEW ANTIBIOTIC

I. Discovery and Biological Activities

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INTRODUCTION

A new antibiotic, porfiromycin, has been detected in fermented broths of Streptomyces ardus sp. n. Porfiromycin is active against a variety of Gram-positive and Gram-negative bacteria in vitro and in vivo. It is also active against KB human carcinoma cells in tissue culture and a variety of experimental tumors in vivo [1]. This paper is concerned with the discovery and the taxonomy of the producing organism, the fermentation conditions necessary for antibiotic production, quantitative paper-chromatographic biological and ultraviolet assays, and the in vitro activity. Companion papers describe the extraction and characterization of porfiromycin [2], the bacterial spectrum and in vivo data [3], and the microbiological assays [4].

TAXONOMY

This new Streptomyces species was isolated from a Missouri soil sample and was checked for identity with the species described in Bergey's "Manual of Determinative Bacteriology" [5] and in Waksman's and Lechevalier's "Actinomycetes and Their Antibiotics" [6]. Since this isolate appears to differ morphologically and physiologically from any previously described species, the name Streptomyces ardus sp. n. is proposed.

This streptomycete is distinguished by its pale-gray aerial mycelium with few monoverticillate and biverticillate sporophores. The culture sporulates with difficulty. A comparison made on Ektachrome indicated that S. ardus differed from the described species in our culture collection. The characteristics of S. ardus on Ektachrome [7] are given in Table 1. The carbon utilization pattern of S. ardus in the synthetic medium of Pridham and Gottlieb [8] is given in Table 2. Additional cultural characteristics are given in Table 3.

Table 1. Appearance of S. ardus on Ektachrome

Agar media	Surface	Reverse
Bennett's	Fair, gray	Tan-brown
Czapek's sucrose	Trace, gray	Colorless
Maltose-tryptone	Fair, gray	Tan-brown
Peptone-iron	Fair, gray	H_2S darkening
Tyrosine (0.1%)	Trace, gray	Tan
Casein-starch	Trace, gray	Tan

FERMENTATION

In view of the limited sporulation of S. ardus, a soil stock was regularly used to inoculate the seed flasks. Seed was grown in 500-ml Erlenmeyer flasks containing 100 ml of broth consisting of cottonseed meal, 10 g/liter and glucose monohydrate (Cerelose), 10 g/liter. The pH was adjusted to 7.0 prior to sterilization. The seed flasks were incubated for 2–3 days on a Gump rotary shaker at 28 or 32 C. This inoculum was used to seed fermentation flasks and/or tanks containing Pabst yeast, 20 g/liter; blackstrap molasses, 20 g/liter; glucose monohydrate, 20 g/liter; and tap water. Prior to sterilization the pH was adjusted to 7.2. The time-course of antibiotic activity and pH of a typical shake flask fermentation are given in Table 4. The fermentation cycles ranged from 2–5 days with no sharp peaks in the yield. The course of the pH was very uniform throughout the fermentation, ranging from 6.0 to 7.0.

Fermentations run from 28–32 C gave optimum yields. When the fermentation was run at 37 C the cycle was shortened 1–2 days and the yield was greatly reduced.

Table 2. Assimilation of Carbon Compounds in Synthetic Medium by S. ardus

Positive	Positive (slight growth)	Negative	Negative (slight growth)
D-galactose	D-fructose	D-xylose	L-arabinose
D-glucose	Soluble starch	Rhamnose	Lactose
D-mannose	Sodium acetate	Sucrose	Cellobiose
Maltose	Sodium citrate	Raffinose	Dextrin
Glycerol		Inulin	Dulcitol
Inositol		Phenol	D-mannitol
		Cresol	D-sorbitol
		Sodium salicylate	Salicin
			Sodium formate
			Sodium oxalate
			Sodium tartrate
			Sodium succinate

ASSAY

A standard agar diffusion assay was devised similar to that of Loo et al., [9] using Difco's brain-heart infusion agar and Staphylococcus aureus FDA-209P as the test organism. This assay is reported in a companion paper [4].

Paper-chromatographic assay. Bio-autographs of the culture filtrate of S. ardus revealed active components which moved in several solvent systems as shown in Fig. 1. A 1 mg/ml standard solution of porfiromycin in methanol was applied at dosages of 1, 2, 5, 10, and 20 μl; on 0.5 by 22.5 in. strips of Schleicher and Schuell No. 589 paper. The assay samples were applied at three dosage levels which were estimated to contain between 1 to 20 μg of porfiromycin. The strips were developed in system V for 16 hr. Two procedures for the detection of the porfiromycin activity on the developed strips were used with equal success and were performed either individually or in conjunction depending on the importance of the sample to be assayed.

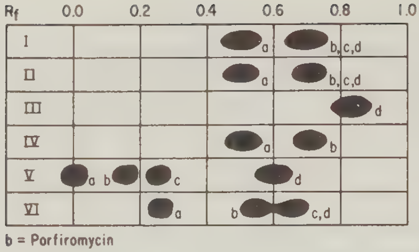
The strips were scanned at 360 mμ by the usual techniques with the Cary recording spectrophotometer (model 11), which had a paper strip attachment [10].

The height of the optical density peak for each level of the standard was measured and plotted on semilogarithmic paper to obtain the dose-response curve. A typical dose-response curve, with the corresponding superimposed optical density peaks recorded by the Cary spectrophotometer from the standard strips, is illustrated in Fig. 2.

For the second assay procedure, which is similar to that of previously described paper-chromatographic assay methods [11], the sections of the strips which contained the porfiromycin component were placed on agar trays seeded with *Sarcina lutea*. A set of standard strips was plated on each tray beside the strips of samples to be assayed, in order to eliminate any error incurred from variable growth of the test organism and the thickness of the agar among trays. The strips were left on the trays during a 37 C incubation period of 16 hr. The strips were removed and the widths of the zones of inhibition were measured

Table 3. Culture Characteristics of *S. ardus*

Medium	Aerial growth	Vegetative growth	Other
Plain gelatin	Good, pale gray	Tan	Tan-brown pigment; trace liquefaction
Nutrient gelatin	Good, pale gray	Tan	Tan-brown pigment; complete liquefaction
Tryptone broth	Fair, gray	Good, gray-tan pellicle; flocculent at base	Tan-brown pigment
Litmus milk	Gray	Good, tan surface ring	No change
Nutrient nitrate broth	Gray	Fair, tan ring; flocculent at base	Tan pigment; nitrate not reduced
Synthetic nitrate broth	None	Fine, colorless to yellow at surface and throughout	Pale yellow pigment; nitrate not reduced
Calcium-malate agar	Trace	Poor, colorless	No pigment
Glucose-asparagine agar	Pink-tan	Fair, pale tan	Trace yellow pigment
Peptone-iron agar	Good, gray	Brown	H_2S darkening
Tyrosine agar (0.1%)	Trace, gray	Poor, gray-brown	Trace, pink-tan pigment
Maltose-tryptone agar	Fair, cottony, cream-white	Good, tan	
Casein-starch agar	None	Good, colorless	Starch incompletely hydrolyzed
Nutrient starch agar	Trace, gray-pink	Good, light brown	Starch incompletely hydrolyzed
Skim milk agar	Good, gray-pink	Good, honey-tan	Yellow pigment; casein hydrolyzed
Bennett's agar			
18 C	Good, lavender-gray	Good, gray-brown	
24 C	Good, lavender-pink	Good, gray-tan	
28 C	Good, lavender-pink	Good, tan	
37 C	Good, maroon	Good, red-brown	
Czapek's sucrose agar			
18 C	None	Fair, colorless	
24 C	Trace, white	Fair, colorless	
28 C	Trace, white	Fair, colorless	
37 C	None	Fair, colorless	



Solvent system	Composition (v/v)
I	1-butanol : water (84 : 16)
II	1-butanol : water (84 : 16) + 0.25% (w/v) p-toluene sulfonic acid
III	1-butanol : glacial acetic acid : water (2 : 1 : 1)
IV	2% piperidine in 1-butanol : water (84 : 16)
V	methanol : benzene : water (1 : 1 : 2)
VI	ethyl acetate saturated with water

Fig. 1. Sarcina lutea bio-autogram pattern of porfiromycin.

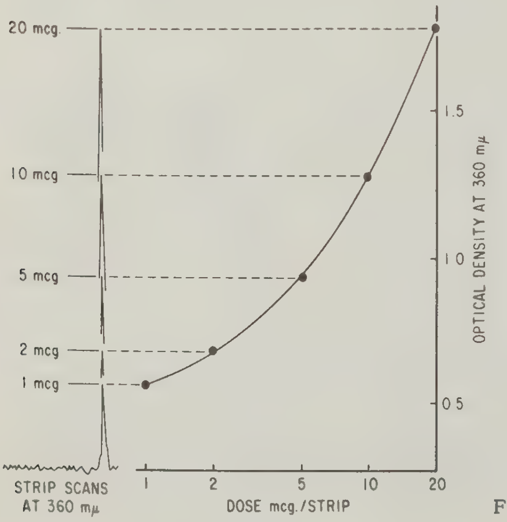
and plotted against logarithmic dose to obtain the dose-response curve. Figure 3 demonstrates a representative dose-response curve.

It should be noted that the "a" component has not been differentiated from mitomycin C by paper chromatography. The preliminary identification of paper chromatograms also indicated a marked similarity of porfiromycin to a second component found in a mitomycin C preparation. The Rf values for porfiromycin and this mitomycin component were identical in solvent systems I–IV. It was not until both were mixed and paper chromatographed in system V that they were found to be separable. Further similarity to the mitomycin family was indicated when developed paper strips of mitomycin C and porfiromycin were scanned for UV absorption. The UV absorption spectrum of porfiromycin corresponds closely to that of mitomycin C.

BIOLOGICAL ACTIVITY IN VITRO

Antibacterial spectrum. A twofold dilution spectrum using yeast extract-peptone broth (peptone, 7.5 g/liter; yeast extract, 2.5 g/liter) was run on porfiromycin. The test organisms, grown for 18 hr at 37 C, were used to inoculate the test medium at a dilution of 1 : 40,000. Observations made after 42 hr of incubation at 37 C and recorded as the minimal inhibitory concentrations are shown in Table 5. These data demonstrate a broad spectrum of activity in vitro.

Tissue culture. The cytotoxicity of porfiromycin to KB human epidermoid carcinoma cells in vitro was determined by the procedure of Smith et al. [12]. At



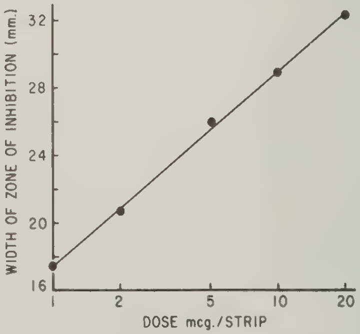


Fig. 3. Porfiromycin dose-response vs. Sarcina lutea.

Fig. 2. UV dose-response curve of porfiromycin.

Table 4. Fermentation of S. ardus in Shaken Flasks

Days	pH	Zone of inhibition, mm	
		S. aureus	K. pneumoniae
2	6.9	29	34
3	6.9	37	27
4	6.9	32	30
5	6.8	30	25

The activity includes porfiromycin and a second component with resembles mitomycin C.

a concentration of 0.5 μg/ml, KB protein synthesis was inhibited 50% (ID_{50}). In vivo antitumor evaluation in animals will be reported in a subsequent paper [1].

Antiprotozoal activity. A broth dilution technique was used to determine the minimal inhibitory concentration, using Bacto-fluid thioglycollate medium plus 5% horse serum (Difco). Porfiromycin inhibited Trichomonas vaginalis at 150 μg/ml and Entamoeba histolytica at 200 μg/ml in vitro.

Antifungal activity. In an agar dilution test porfiromycin demonstrated activity at 100 μg/ml against Nocardia asteroides but was inactive at 1000 μg/ml against a variety of other fungi (Table 6).

Porfiromycin is not cross resistant with the following antibiotics: penicillin, novobiocin, streptomycin, chloromycetin, erythromycin, tetracycline, and neomycin.

Table 5. Antibacterial Spectrum of Porfiromycin

Test organism	Minimal inhibitory concentration μg /ml
Diplococcus pneumoniae Felton I*	0.025
Klebsiella pneumoniae A-D	0.1
Pasteurella multocida 449	25
Proteus vulgaris ATCC 6380	6.25
Pseudomonas aeruginosa ATCC 9027	25
Salmonella paratyphi B 4	12.5
Salmonella pullorum 75	1.56
Salmonella typhi TG3	6.25
Staphylococcus aureus 284	0.1
Staphylococcus albus ATCC 151	0.1
Streptococcus fecalis ATCC 6057	0.2
Streptococcus hemolyticus C 203*	0.025
Streptococcus viridans 25-11	0.025
Aeromonas liquefaciens*	0.78
Aeromonas salmonicida*	0.20

*Brain-heart infusion—Difco, 37 g/liter.

Table 6. Antifungal Spectrum of Porfiromycin

Test organism	Minimal inhibitory concentration, μg/ml
Nocardia asteroides*	100
Blastomyces dermatitidis	>1000
Coccidioides immitis	>1000
Phialophora verrucosa	>1000
Cryptococcus neoformans	>1000
Histoplasma capsulatum	>1000
Sporotrichum schenckii	>1000
Monosporium apiospermum	>1000
Candida albicans Abbott	>1000
Microsporum canis	>1000
Trichophyton interdigitale	>1000
Trichophyton rubrum	>1000

*Fungal spectrum agar in g/liter of distilled water: dextrose, 10; Bacto-peptone, 5; yeast extract, 1; bacto agar, 20. Adjust pH to 6.8.

ACKNOWLEDGMENT

We wish to acknowledge the cooperation of Mr. W. A. Murphy for his assistance with fermentation, Dr. J. E. Grady for tissue culture assay, and Dr. R. M. Michaels for antiprotozoal data.

SUMMARY

A new broad-spectrum antibiotic, porfiromycin, was discovered in the culture filtrate of Streptomyces ardus sp. n. The morphology, physiology, and fermentation conditions of this new species are described. Quantitative paper chromatographic, biological, and ultraviolet assays are described.

Porfiromycin is active in vitro against a variety of Gram-positive and Gram-negative bacteria and KB carcinoma cells. It is marginally active against protozoa and shows slight activity against fungi.

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PORFIROMYCIN, A NEW ANTIBIOTIC

II. Isolation and Characterization

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INTRODUCTION

This paper describes the isolation and purification of a new broad-spectrum antibiotic, porfiromycin, from a Streptomyces ardus fermentation broth. Chemical and physical properties of the crystalline material are described. Biological properties of porfiromycin are described in companion papers [1-4].

ISOLATION

Crystalline porfiromycin was isolated by the following procedure. The whole broth was filtered at pH 7 and the filtrate was extracted with 1-butanol. The butanol extract was concentrated by azeotropic distillation under reduced pressure with the addition of water to obtain an aqueous solution. This solution was then extracted with chloroform and the chloroform extracts were evaporated to dryness. Subsequent investigations showed that direct chloroform extraction of the filtered broth also gave satisfactory recovery of the activity.

Crude preparations thus obtained contain several activities in addition to porfiromycin, two of which have been tentatively identified as mitomycins A and C. Also present, and in considerable quantity, is an antibacterially inactive material, identified as cinnamic amide. These substances are separable by partition chromatography on diatomaceous earth (Fig. 1) or by adsorption chromatography on alumina. The partition columns were run using solvent systems consisting of mixtures of ethyl acetate, cyclohexane and pH 5.0 McIlvaine's buffer. Final purification was achieved by countercurrent distribution between ethyl acetate and water.

CHARACTERIZATION

Porfiromycin is obtained by the above methods as dark purple triclinic crystals which melt with decomposition at 201-201.5 C. Single crystal x-ray measurements indicate the molecular weight to be 344. Although titrations in aqueous solution show no acidic or basic groups, titrations with perchloric acid in glacial acetic acid indicate an equivalent weight of 344. Results of elemental analyses best fit the empirical formula $C_{16}H_{20}N_4O_5$ (molecular weight 348).

The ultraviolet absorption spectrum of porfiromycin (Fig. 2) is indistinguishable from that of mitomycin C, showing maxima in water at 217 $m\mu$ ($a = 72$) and 364 $m\mu$ ($a = 69$), with a shoulder at 248 $m\mu$ ($a = 25$). The visible spectrum shows a broad maximum at 575 $m\mu$ ($a = 0.70$). The infrared spectrum of porfiromycin in mineral oil suspension (Fig. 3) shows identifying absorptions at the following frequencies: 3370, 3250, 3150 (OH/NH), 3000 (shoulder), 1720, 1690 (C=O), 1640,

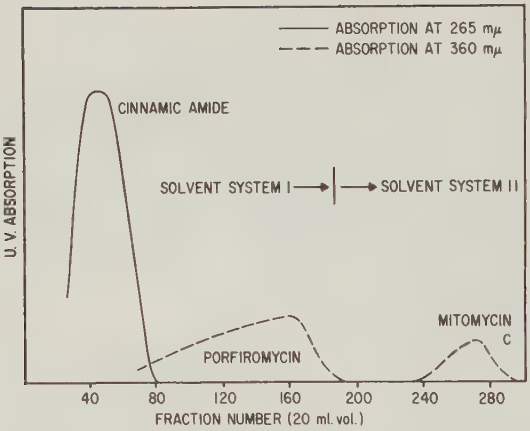

Fig. 1. Partition chromatography of porfiromycin crude preparations.

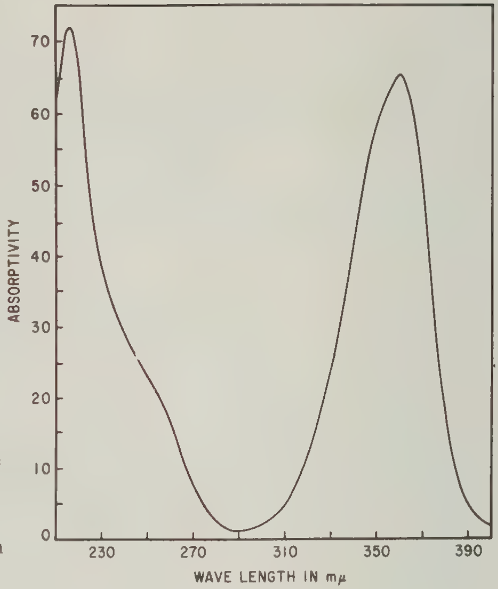

Fig. 2. Ultraviolet absorption spectrum of porfiromycin in water.

1600, 1555, 1535 (C=N, C=C), 1440, 1403, 1390, 1365, 1342, 1320, 1245, 1215, 1160, 1135, 1098, 1065, 1045, 1028, 1008, 975, 952, 918, 890, 848, 808, 782, 758, 729, 718, 692, and 675 cm^{-1}. The infrared spectra of porfiromycin and mitomycin C show differences, and the compounds are readily separable by paper chromatography [1].

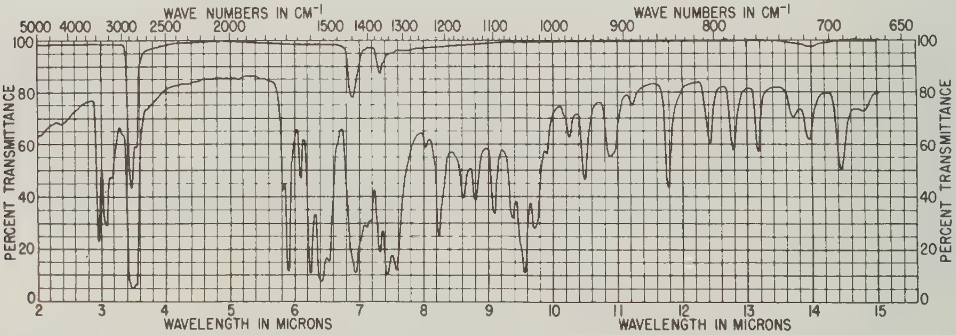

Fig. 3. Infrared absorption spectrum of porfiromycin in mineral oil suspension.

Because of the intense color of porfiromycin solutions, optical rotation measurements must be made on very dilute solutions. Under these conditions $[\alpha]_D^{25} = +275 \pm 55$ deg (c, 0.1% methanol). The rotatory dispersion curve (Fig. 4) shows a maximum at 430 mμ, with $[\alpha]_{430}^{25} = +2525 \pm 50$ deg.

Porfiromycin is slightly soluble in water, moderately soluble in polar organic solvents, and essentially insoluble in hydrocarbon solvents.

Dry crystalline porfiromycin stored at 70 C showed no detectable degradation after six months. Both acid and alkaline solutions deteriorate rapidly. Stability studies in solution will be reported in detail elsewhere [5].

EXPERIMENTAL

Isolation from culture broth. 219 liters of culture broth was filtered at pH 6.8 using 5% (w/v) Dicalite 4200 filter-aid and the filtrate was extracted twice with 70-liter portions of 1-butanol. The extracts were combined and concentrated azeotropically in vacuo, with the addition of water, to an aqueous solution which was freeze dried. The dry material weighed 38 g. From this preparation, 17.8 g was dissolved in 100 ml of water and the aqueous solution was extracted three times with 100-ml portions of chloroform. The combined chloroform extracts were concentrated in vacuo to dryness to give 6.4 g of residue.

Partition chromatography. Solvent system I (Fig. 1) consisted of 5 parts of ethyl acetate, 10 parts of cyclohexane, and 1 part of pH 5.0 McIlvaine's buffer. 107 g of Celite 545 was mixed with 400 ml of upper phase and, while stirring, 60 ml of lower phase was added. The mixture was poured into a $1\frac{1}{4}$-in.-diameter glass column and packed with 2 lb/in.^{2} air pressure to a height of 15 in. The crude material, obtained as described above (6.4 g), was dissolved in 8 ml of lower phase. The solution was mixed with 6 g of Celite 545 and 50 ml of upper phase, and packed onto the top of the prepared column.

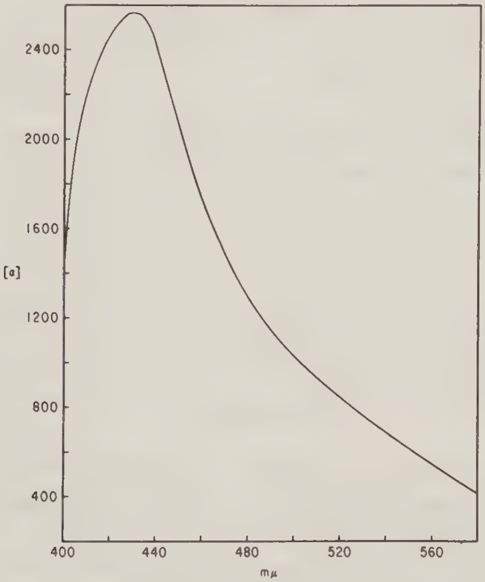

Fig. 4. Rotatory optical dispersion of porfiromycin in methanol.

The column was eluted with upper phase at the rate of 4 ml/min and 20-ml fractions were collected. The column was monitored by the determination of solids, by ultraviolet absorption, and by spotting dipped filter-paper disks on agar trays seeded with S. aureus.

Cinnamic amide was obtained from early fractions as a white crystalline solid, mp 144–148 C. Comparison with an authentic sample prepared from cinnamic acid confirmed the identity.

Fractions 81–170 contained porfiromycin. These were pooled, concentrated to dryness in vacuo, and the residue was dissolved in 25 ml of methanol. The solution was washed three times with 20 ml of Skellysolve B and the methanol phase was evaporated to dryness to give 0.9 g of amorphous material.

After elution of the porfiromycin from the column, the eluant was changed to the upper phase of solvent system II, consisting of five parts ethyl acetate, five parts of cyclohexane, and one part of pH 5 McIlvaine's buffer. Using this system another activity was eluted and isolated as an amorphous purple solid. Paper chromatography tentatively identified this activity as mitomycin C.

Countercurrent distribution and crystallization. The crude porfiromycin (0.9 g) obtained by partition chromatography was countercurrently distributed for 700 transfers between equal volumes of ethyl acetate and water. Fractions 404–434 (K =1.57) were pooled and concentrated to dryness under reduced pressure. The residue was dissolved in 20 ml of ethyl acetate and filtered and evaporated at room temperature to a volume of 6 ml, at which point crystallization began. The mixture was cooled to 0 C and the crystals were collected by filtration. After vacuum drying at room temperature, 233 mg of porfiromycin was obtained as dark purple crystals.

Analysis. Calculated for $C_{16}H_{20}N_4O_5$: C, 55.16; H, 5.79; N, 16.09; O, 22.96. Found: C, 54.96, 55.23; H, 5.92, 5.99; N, 16.19; O, 22.17, 22.60.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to various members of The Upjohn Company who contributed to this work. In particular we wish to thank Dr. J. H. Ford, Mr. F. L. Cunningham, and associates for the large-scale preparations; Mr. M. F. Grostic for infrared spectra; Dr. R. W. Rinehart for ultraviolet and visible spectra; Mr. W. A. Struck and associates for microanalyses; Mr. J. A. Fox for countercurrent distributions; and Dr. J. W. Shell for crystallography.

SUMMARY

The isolation and characterization of a new broad-spectrum antibiotic, porfiromycin, have been described. Analytical results on crystalline material suggest the empirical formula $C_{16}H_{20}N_4O_5$.

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PORFIROMYCIN, A NEW ANTIBIOTIC

III. In Vitro and In Vivo Evaluation

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INTRODUCTION

Porfiromycin, a new antibiotic recently described by DeBoer et al. [1], was reported to be active against numerous Gram-positive and Gram-negative organisms. The methods of its isolation, as well as its physical, chemical, and biological properties, are reported in other studies [1,2,3]. It is the purpose of the present paper to report in more detail on the antimicrobial and biological properties of this antibiotic.

MATERIALS AND METHODS

In vitro. The sensitivity of bacteria to porfiromycin was determined by a standard twofold tube-dilution method [4] in brain-heart infusion broth (Difco). The antibiotic was dissolved in sterile distilled water at pH 6.6. End-point determinations were read by visual inspection after 18 hr incubation at 37 C.

Studies designed to compare the bacteriostatic and bactericidal end points were carried out in the same medium and under the same conditions as the in vitro sensitivity tests. The bactericidal end points were determined by removing 0.1 ml from those tubes containing no visible growth, transferring it into 5.0 ml of sterile brain-heart infusion broth, and incubating the subculture for an additional 24 hr.

The effect of human and rabbit serum, and human urine, on the inhibitory properties of porfiromycin was also determined in brain-heart infusion broth.

In vivo. Male white mice, CF-1, 18–20 g (unless otherwise specified), were selected at random from animal pools for use in the animal-protection tests. Techniques for testing compounds in infected mice have been described previously [5]. Chronic kidney infections [6] in mice were produced by the intravenous injection of 0.2 ml containing 10^6 to 10^7 cells of an 18-hr broth culture of the Smith strain of Staphylococcus aureus. Experiments in which porfiromycin was used in Leptospira pomona infections were designed to demonstrate its effectiveness in the Mongolian gerbil (Meriones unguiculatus) [7]. In this species of rodent an acute, fatal leptospirosis was produced by the intraperitoneal injection of 1000 cells. The injection of less than 1000 cells produced a chronic, progressive infection resulting in a urinary carrier state.

Freshly prepared stock solutions of the antibiotic were made at the beginning of each experiment in phosphate buffer adjusted to pH 7.0 and stored in amber bottles. The antibiotic concentration was such that the desired dose was contained in 0.2 ml when administered subcutaneously or in 0.5 ml when administered by gastric intubation. In the mouse-protection test the infecting dose of

Table 1. The Antibacterial Spectrum* of Porfiromycin

Organism	Strain No.	End point, µg/ml	
		Porfiromycin	Chloramphenicol
Staphylococcus aureus	76	0.19	3
	391	0.39	3
	488	0.39	100
	595	0.19	0.39
	599	0.39	–
	601	0.19	100
	607	0.09	1.5
	703	0.09	3
	704	0.78	3
	738	0.39	6
	739	0.78	12
	740	0.39	6
	749	0.39	–
Diplococcus pneumoniae†	41	3.1	–
	42	3.1	0.78
	43	25	0.78
	44	6.2	0.39
	190	0.78	–
Streptococcus hemolyticus	752	0.78	0.78
	753	1.5	–
	754	0.09	0.78
	755	0.19	0.39
	757	1.5	–
	758	0.09	–
	759	0.09	1.5
	778	0.19	–
	779	0.19	–
	780	0.39	0.39
	769	0.19	–
	152	0.04	1.5
Streptococcus fecalis	700	1.5	12
	701	6.2	6
	157	12.5	6
Aerobacter aerogenes	437	12.5	–
	438	3.1	–
	441	1.5	–
	445	12.5	–
Escherichia coli	311	25	3
	422	25	12
	527	12.5	3
	557	12.5	–
Proteus morganii	340	6.2	12
	341	6.2	25
	346	6.2	>100
Proteus rettgeri	339	6.2	6
	342	25	12
	344	100	–

Table 1 (continued)

Organism	Strain No.	End point, μg/ml	
		Porfiromycin	Chloramphenicol
Proteus vulgaris	232	50	25
	338	50	–
	343	50	100
Pseudomonas aeruginosa	348	50	–
	350	50	100
	351	25	100
	400	50	–
Pasteurella multocida	264	0.78	0.04
Alcaligenes fecalis	4	50	–
Salmonella enteridis	113	12.5	–
	114	25	1.5
Salmonella gallinarum	145	12.5	0.78
	265	12.5	–
Salmonella paratyphi A	260	3.1	0.78
Salmonella paratyphi B	262	25	3
Salmonella pullorum	267	1.5	1.5
Salmonella typhosa	105	25	–
	215	3.1	–
	643	3.1	–
Shigella dysenteriae	139	12.5	6
Shigella paradysenteriae	134	25	0.78
Shigella flexneri	143	1.5	0.78
	144	12.5	–
Klebsiella pneumoniae	58	1.5	–
	272	25	50
Neisseria catarrhalis‡	87	3.1	0.78
	88	0.19	0.78
	89	6.2	–
Neisseria gonorrhoeae§	3065	0.19‖	–
Clostridium perfringens	247	25	12
Clostridium sporogenes	72	0.19	12
Clostridium tetani	680	0.78	50

*The medium used was brain-heart infusion broth and end points were determined after 18 hr incubation at 37 C unless specified otherwise.
†Tested in 7AW broth.
‡Tested in Dextrose Starch agar.
§G. C. starch medium
‖48-hr end point.

organism and the drug were administered so that the drug was given immediately following the bacterial challenge. Therapy was continued for four days and the mice were observed for an additional three days. At the end of seven days the median protective dose (CD_{50}) of the antibiotic was calculated from mortality rates in groups of ten mice which had been challenged with 100 LD_{50} of organism. In experiments concerned with S. aureus and Leptospira chronic kidney infections porfiromycin was administered daily, subcutaneously or orally, for 13 or 10 days (Leptospira). On the fourteenth day after infection the animals were sacrificed. The kidneys from surviving animals were removed and ground in Pyrex tissue grinders containing 4 ml brain-heart infusion broth (Difco). The presence of viable Leptospira in the organ homogenate was determined by transferring 0.05 ml of the homogenate into 5.0 ml Schuffner's medium supplemented with 10% rabbit serum and incubating at 30 C for 28 days. The culture was examined periodically under dark-field illumination for the presence of Leptospira. The number of viable organisms in kidneys from mice infected intravenously with S. aureus Smith was determined by plating 0.1 ml of the organ homogenate, diluted in tenfold increments, on the surface of agar plates. After incubation, suitable plates were examined and the number of bacteria expressed as the colony count per gram of kidney. Evaluation of the antibiotic was based on the absence of Leptospira in the kidneys and on the reduction of the number of S. aureus in kidneys from treated mice as compared to the number in kidneys from untreated control mice.

RESULTS

In vitro. Porfiromycin possessed good activity against micrococci, streptococci, diplococci, enterococci, and Pasteurella, Salmonella, Clostridium, and Neisseria species. It also was active against species of Aerobacter, Klebsiella, Proteus, Pseudomonas, and coliform microorganisms, but wide variations in sensitivity were observed within species of each genus (Table 1). The end points listed were determined after 16-18 hr incubation unless otherwise specified. Further incubation up to 48 hr did not change the end points appreciably, indicating that porfiromycin was bactericidal rather than bacteriostatic.

One of the techniques used to determine the bactericidal vs bacteriostatic activity of porfiromycin was the subculture procedure, i.e., first determining the in vitro end points by the previously described methods, and then removing 0.1 ml from each tube showing no growth and placing it into 5.0 ml brain-heart infusion broth. A lack of growth in such a subculture after 48 hr at 37 C was considered the bactericidal end point. Results of this study are presented in Table 2 and demonstrate that porfiromycin compares favorably with tetracycline, erythromycin, penicillin, and novobiocin as a bactericidal agent. The bactericidal vs the bacteriostatic end points vary between two- and tenfold, depending on the test organism. Viable count determinations made under the same conditions reveal that 1.5 $\mu g/ml$ of the antibiotic reduces the viable population of S. aureus below a countable level in 4 hr (Fig. 1).

Experiments designed to test the effect of urine and human or rabbit serum on the in vitro activity of porfiromycin demonstrated that concentrations of either human or rabbit serum as high as 50% had no effect on the in vitro activity of the antibiotic against S. aureus or Proteus vulgaris. 50% concentrations of pooled human urine reduced the activity of the antibiotic about fourfold when tested against S. aureus but did not alter the end point when P. vulgaris was used (Table 3).

Table 2. Comparison of the Bactericidal and Bacteriostatic* Activity of Porfiromycin to Other Antibiotics

Organism	Strain No.	Tetracycline, μg/ml		Erythromycin, μg/ml		Penicillin, μg/ml		Novobiocin, μg/ml		Porfiromycin, μg/ml	
		Bacterio-static	Bacteri-cidal	Bacterio-static	Bacteri-cidal	Bacterio-static	Bacteri-cidal	Bacterio-static	Bacteri-cidal	Bacterio-static	Bacteri-cidal
S. aureus	76	0.78	6.2	0.78	12.5	0.39	3.1	12.5	12.5	0.78	6.2
S. aureus	703	0.78	6.2	100†	>100	0.19	3.1	1.5	12.5	0.78	6.2
S. hemolyticus	752	0.78	6.2	0.09	1.5	0.39	0.78	6.2	12.5	1.5	3.1
S. hemolyticus	758	6.2	25	0.19	1.5	0.09	0.78	12.5	>100	0.09	0.09
E. coli	311	3.1	100	50	>100	50	50	>100	>100	50	50
P. morganii	340	6.2	100	>100	>100	>100	>100	>100	>100	6.2	12.5
P. aeruginosa	95	12.5	100	>100	>100	>100	>100	>100	>100	25	50

*Expressed as the minimal inhibitory concentration (MIC) in μg/ml.
†Erythromycin-resistant strain.

Table 3. Effect In Vitro of Urine and Human or Rabbit Serum on Porfiromycin Activity

Organism	Strain No.	% Urine or serum	18-hour end point, μg/ml		
			Urine	Human serum	Rabbit serum
Staphylococcus aureus	76	50	6.2	0.78	0.78
	76	25	6.2	0.78	0.78
	76	10	3.1	0.78	0.39
	76	5	3.1	0.78	0.39
	76	1	1.5	0.78	0.78
	76	0	1.5	0.78	0.39
Proteus vulgaris	232	50	50	25	>100
	232	25	50	25	>100
	232	10	50	50	>100
	232	5	50	50	>100
	232	1	50	50	>100
	232	0	50	50	>100

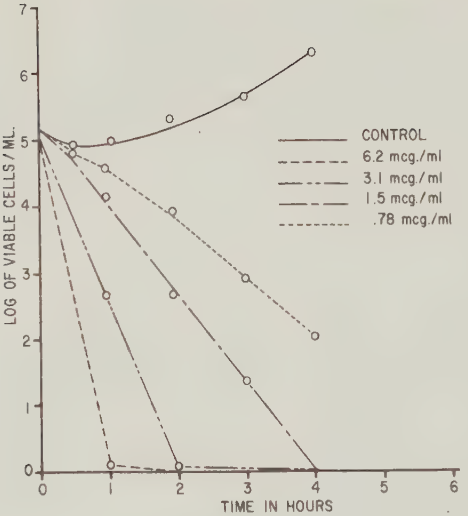

Fig. 1. The bactericidal effect of porfiromycin on Staphylococcus aureus.

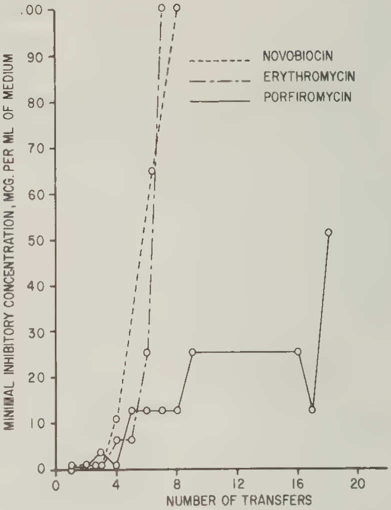

Fig. 2. Rate of development of resistance to porfiromycin and other antibiotics by Staphylococcus aureus.

Attempts to demonstrate cross resistance of porfiromycin with any of the commonly used antibiotics were negative. The results in Table 4 are from experiments in which a series of clinical isolates with varying antibiotic-resistance patterns and laboratory-developed resistant strains were tested.

The rate of resistance development of S. aureus UC-76 to the antibiotic was studied by using the standard twofold dilution technique and employing brain-heart infusion broth as the test medium. The end points were determined after 18 hr incubation at 37 C. The last tube showing growth was used as a source of inoculum for the next dilution series. Results are presented graphically in Fig. 2. The data show that S. aureus develops resistance to porfiromycin more slowly than to erythromycin or novobiocin.

In vivo. Porfiromycin is highly effective in protecting mice (Table 5) infected with Streptococcus hemolyticus, Streptomyces viridans, S. aureus, Diplococcus pneumoniae, Klebsiella pneumoniae, Pasteurella multocida, and Salmonella typhimurium. It was also effective in protecting 4-day chicks against lethan challenges of Salmonella gallinarum. The antibiotic is inactive in mice infected with Pseudomonas aeruginosa, P. vulgaris, Escherichia coli, Salmonella paratyphi B, Shigella sonnei, and Mycobacterium tuberculosis. It was also inactive against Trichomonas vaginalis in mice dosed at 6 mg/kg/day subcutaneously and Endamoeba histolytica in rats dosed orally at 6.2 mg/kg/day. Median protective doses (CD_{50}) required to protect infected animals ranged from 0.09 to 4.0 mg/kg/day subcutaneously and 0.12 to 3.8 mg/kg/day orally.

It is interesting to note that porfiromycin shows about the same degree of activity in mice when administered either parenterally or orally. These results indicate a high degree of oral absorption. In addition, porfiromycin was effective in protecting chicks infected with S. gallinarum when the antibiotic was given subcutaneously but not when administered orally, perhaps illustrating the instability of the antibiotic in an environment as acid as the crop of a chicken (pH 4.0). The data in Table 5 also demonstrate that porfiromycin is effective

Table 4. Effect* of Porfiromycin on Staphylococci Having Varied Resistance Patterns

Strain No.	Resistance pattern	18-hr end point, μg/ml
	Clinical Isolate	
488	Penicillin, chloramphenicol, and tetracycline	0.39
391	Penicillin, chloramphenicol, streptomycin, and tetracycline	0.39
740	Penicillin and tetracycline – belongs to phage type 80/81	0.39
738	Penicillin, tetracycline, and erythromycin – phage type 80/81	0.39
749	Penicillin, chloramphenicol, erythromycin, tetracycline, and streptomycin	0.39
76	Control strain†	0.19
	Laboratory Strain	
607	Novobiocin	0.09
601	Neomycin C	0.19
599	Neomycin A	0.39
703	Erythromycin	0.09
595	Streptolydigin	0.19

*Expressed as the minimal inhibitory concentration (MIC) in μg/ml.
†This strain previously unexposed to antibiotics.

Table 5. Activity of Porfiromycin in Experimentally Infected Animals*

Organism	Challenge, LD_{50}†	Median protective dose, CD_{50}‡	
		Subcutaneous	Oral
S. hemolyticus C2O3	81	0.3 (0.2-0.4)	0.35 (0.26-0.44)
S. viridans 871	151	0.3 (0.2-0.4)	0.58 (0.33-0.83)
S. aureus 284§	83	0.69 (0.37-1.01)	1.7 (1.3-2.1)
S. aureus 607§	166	0.46 (0.33-0.59)	2.7 (2.0-3.4)
S. aureus 3090§	309	1.2 (0.8-1.6)	3.8 (2.7-4.9)
S. aureus 3067§	12	0.2 (0.1-0.3)	0.6 (0.2-1.0)
D. pneumoniae I	468	1.7 (1.4-2.0)	2.6 (2.0-3.2)
D. pneumoniae III	100	0.3 (0.2-0.4)	0.67 (0.48-0.86)
K. pneumoniae A-D	68	1.26 (0.97-1.55)	2.3 (1.5-3.1)
P. multocida 449	100	2.0 (1.9-2.1)	2.4 (2.0-2.8)
P. aeruginosa YED	123	>8.0	>20
P. vulgaris 347	34	>8.0	>20
E. coli 311	148	>8.0	>20
S. paratyphi B No. 4	51	>8.0	>20
S. typhimurium SB682	575	0.09 (0.03-0.15)	0.12 (0.07-0.17)
S. gallinarum 8410	10	4.0 (2.6-5.4)	>160
S. sonnei II 933	4	>8.0	>20
M. tuberculosis H37Rv	–	>6.0	>12

*Leghorn 4-day chicks were used in the S. gallinarum infection. In all others CF-1 mice weighing 18-20 g were used.
†Median lethal dose.
‡Expressed as mg/kg/day with 95% confidence limits.
§S. aureus 284—sensitive, 607—novobiocin-resistant, 3090—streptomycin-resistant, 3067—penicillin-resistant.

not only in controlling infections produced by S. aureus, but is active against S. aureus strains resistant to other antibiotics.

Data illustrating the effectiveness of porfiromycin on chronic S. aureus Smith infections are presented in Table 6. All drug levels produced a therapeutic response and the two highest drug levels (8 and 4 mg/kg) eradicated all organisms from the kidneys. In a similar infection produced by Streptomyces zymogenes (fecalis) the antibiotic was not effective. The highest drug level used (Table 7) produced a reduction in the microbial population of the kidneys but failed to completely clear them of bacterial cells.

The results of a series of experiments in which porfiromycin was used as a chemotherapeutic agent in experimental Leptospira pomona infections have shown that the antibiotic altered these infections to a limited extent. Data in Table 8 demonstrate that the antibiotic offered a fair degree of protection to gerbils infected with a lethal dose of L. pomona when the material was administered by either the parenteral or oral route. Toxicity is evident, however, at the highest subcutaneous level (6 mg/kg), with the death pattern evident here being typical for gerbils and mice in that the majority of the animals die during the second week of dosing. If the toxicity deaths are disregarded in the parenterally treated group, the antibiotic elicits a more typical therapeutic dose-response in the subcutaneously-treated gerbils than in the orally-treated groups. Evidence of parenteral activity is also present in the results presented in Table 9. These

Table 6. Effect of Subcutaneous Administration of Porfiromycin on a Chronic S. aureus Infection*

Drug level,† mg/kg/day	Number of mice with viable cells in kidneys‡	Average bacterial count $\times 10^8$
8	0/10	–
4	0/10	–
2	5/10	0.06
1	4/10	0.02
0.5	7/10	0.71
0	9/9	4.9

*Mice were infected intravenously with 0.2 ml 1:1 dilution of 18-hr culture of Smith strain.
†Mice dosed daily for 13 days, sacrificed on fourteenth day.
‡Number of positive mice/total number surviving.

Table 7. Effect of Subcutaneous Administration of Porfiromycin on a Chronic S. zymogenes (fecalis) Infection*

Drug level,† mg/kg/day	Number of mice with viable cells in kidneys‡	Average bacterial count $\times 10^8$
8	6/10	0.52
4	8/10	0.47
2	6/9	0.97
1	8/10	3.2
0.5	9/10	3.0
0	10/10	4.42

*Mice were infected intravenously with 0.2 ml. 1:1.5 dilution of 18-hr culture of S. zymogenes (fecalis).
†Mice dosed daily for 13 days, sacrificed on fourteenth day.
‡Number of positive mice/total number surviving.

Table 8. Effect[*] of Porfiromycin on a Lethal Experimental L. pomona Infection[†] in Gerbils

Route	Drug level,[‡] mg/kg/day	Day of death											Mortality ratio[§]
		4	5	6	7	8	9	10	11	12	13	14	
Subcutaneous	6		1			2	2	4					9/9
	3							1					1/9
	1.5		1	2	1	1							5/9
	0.75		5	2	2								9/9
	0.375		4		2		1						7/9
Oral	15				1	1							2/9
	7.5			3									3/9
	3.75			1	1								2/9
	1.875			2	1		1						4/9
	0.937		1	1									2/9
	0		4	4	1								9/9

[*] Measured as increased survival time.
[†] Gerbils were infected i.p. with about 1000 cells from a 10-day culture grown in Schuffner's medium at 30 C.
[‡] Animals received daily doses for ten consecutive days, starting on day of infection.
[§] Number dead/total number.

data summarize an experiment designed to test the efficacy of porfiromycin in L. pomona carriers. As can be seen, the challenge dose of Leptospira did not kill the untreated gerbils. Kidney cultures from this group show them to be uniformly shedding the organism. Orally administered porfiromycin did not completely eradicate the carrier state, although some beneficial effect can be noted at all treated levels. Parenterally administered, the antibiotic was effective at 4 and 2 mg/kg/day. Toxicity was again evident at the top level (8 mg/kg).

Table 9. Effect[*] of Porfiromycin on a Chronic Experimental L. pomona Infection[†] in Gerbils

Route	Drug level,[‡] mg/kg/day	Day of death											Mortality ratio[§]	Number of gerbils with culturable leptospiras[‖]
		4	5	6	7	8	9	10	11	12	13	14		
Subcutaneous	8		1		2			4		2			9/9	–
	4												0/9	0/4
	2												0/9	0/4
	1												0/9	2/4
	0.5												0/9	3/4
Oral	20	1								1			2/9	3/4
	10												0/9	2/4
	5												0/9	1/4
	2.5												0/9	3/4
	1.25												0/9	4/4
	0				1	1			1				3/10	3/3

[*] Measured as increased survival and on clearing of kidney.
[†] Gerbils were infected i.p. with about 1000 cells. Culture low in virulence and induced carrier state only.
[‡] Animals received daily doses for ten consecutive days, starting on day of infection.
[§] Number of dead/total number.
[‖] Four animals of survivors sacrificed and kidneys cultured for leptospiras. Figure represents number of positive cultures/number of animals cultured.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Mr. D. F. Schwartz and Mr. D. Blum for their technical assistance in this work and to Dr. A. L. Erlanson of Parke, Davis and Company for supplying information and the culture of S. zymogenes.

SUMMARY

Data have been presented that illustrate good in vitro and in vivo activity of porfiromycin against a wide variety of both Gram-positive and Gram-negative organisms.

Resistance in vitro to porfiromycin by Staphylococcus aureus developed slowly and with difficulty. There was no evidence of cross resistance either with laboratory-developed resistant strains or with resistant cultures from clinical sources.

Porfiromycin was effective against a variety of infections in several species of animals. It was active when administered by either oral or parenteral routes and its activity was not altered by the presence of human or rabbit serum or human urine.

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PORFIROMYCIN, A NEW ANTIBIOTIC

IV. Microbiological Assays

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From a variety of microorganisms susceptible to porfiromycin [1–3] several strains of Staphylococcus aureus appeared to be most suitable as assay organisms. Our experience in the past has indicated that assays of different sensitivities are desirable to suit different assay problems. For control purposes when using purified drugs a sensitivity of 1–2 μg/ml is quite satisfactory, and in clinical studies involving blood level determinations, an assay which will detect concentrations of 0.1–0.2 μg/ml of drug is adequate. In certain situations, however, such sensitivities are not sufficient. For instance, for the detection of possible drug residues in animal tissues, an assay sensitivity of 0.01 or 0.001 μg/ml is highly desirable. Several assays for porfiromycin, developed with sensitivities ranging from 0.75 to 0.001 μg/ml, are described in this paper.

MATERIALS AND METHODS

Disc-plate assay with Staphylococcus aureus FDA-209P. Brain-heart infusion agar was inoculated with an 18- to 20-hr culture grown in Penassay * seed broth on the reciprocating shaker at 37 C at the rate of 0.3 ml of the culture per 100 ml agar. The seeded medium was dispensed into 100 × 20 mm plastic petri dishes† in 8-ml portions, and the agar was allowed to solidify. A standard stock solution was prepared by dissolving 50 mg of porfiromycin in 2 ml of methanol and diluting to a concentration of 1 mg/ml with pH 7, 0.1 M phosphate buffer. Stock solutions have been kept in the refrigerator for at least two months without any detectable loss of potency. Working standard solutions were prepared by diluting the stock solution with pH 7 phosphate buffer to concentrations of 12.5, 6.25, 3.125, 1.56, and 0.78 μg/ml. Each solution was applied to one paper disc (12.7 mm) on each of eight replicate plates, and the plates were incubated overnight at 30 C. The diameters of the zones of inhibition were measured and the potencies of the tested samples were estimated from a standard curve plotted as logarithm of dose versus zone diameter.

Disc-plate assay with Staphylococcus aureus UC-607 on nutrient agar. The assay medium was prepared by inoculating 0.2 ml of a 6-hr culture, grown in brain-heart infusion broth (Difco) on the reciprocating shaker at 37 C, into 100 ml of nutrient agar. The concentrations used for the standard curve were 0.08, 0.04, 0.02, and 0.01 μg/ml.

In all other respects the assay technique was identical with that described above.

Turbidimetric assay with Staphylococcus aureus UC-607. The assay medium was prepared by inoculating 0.2 ml of a 6-hr culture grown in brain-heart infusion broth at 37 C on a reciprocating shaker, into 100 ml of nutrient broth.

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*Difco, Detroit, Michigan.
†Falcon Plastic, Culver City, California.

Working stock solutions were prepared at concentrations of 1.0 and 0.1 μg/ml by diluting the 1-mg/ml stock solution with pH 7 phosphate buffer. The standards were prepared by adding the required amount of the stock solutions to seeded nutrient broth to attain concentrations of 0, 0.001, 0.003, 0.009, and 0.027 μg/ml. These solutions were aseptically dispensed in triplicate into sterile, 18 × 150 mm, optically calibrated test tubes in 10-ml portions. The tubes were incubated in a water bath at 37 C for 16–18 hr and the optical density measured on a Beckman B spectrophotometer at 650 mμ. The standard curve was made by plotting the logarithm of the drug concentration against optical density.

RESULTS AND DISCUSSION

Typical dose-response curves of the three assays described are presented in Figs. 1–3. The assay using S. aureus FDA-209P on brain-heart infusion agar (Fig. 1) has a sensitivity of about 0.75 μg/ml and gives very sharp zones. This assay is being used to evaluate fermentation beers and pure samples of the drug. The sensitivity can be increased further to about 0.2 μg/ml by cooling the assay plates for 2–3 hr at 5 C after the antibiotic is applied. This sensitivity is sufficient for blood level studies.

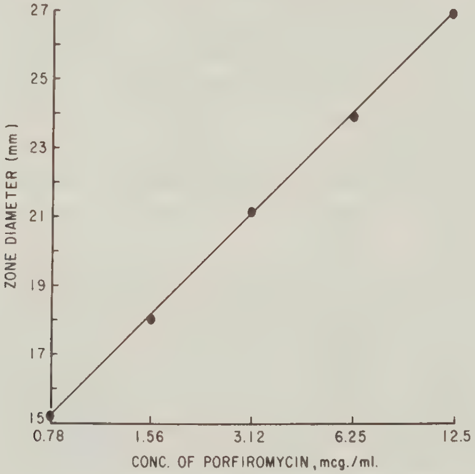

Fig. 1.

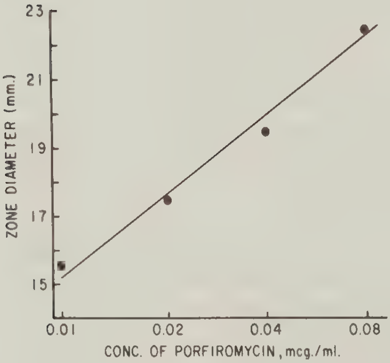

Fig. 2.

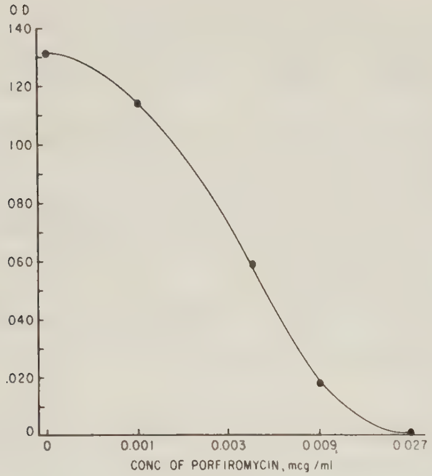

Fig. 3.

The assay using S. aureus UC-607 on nutrient agar has a sensitivity of 0.02 μg/ml, as can be seen in Fig. 2. However, the zones have somewhat hazy edges, which is undesirable for the assay of samples where such sensitivity is not required.

A typical dose-response curve of the turbidimetric assay is presented in Fig. 3. This is an extremely sensitive assay which can detect a drug concentration as low as 0.001 μg/ml. These very sensitive assays are required for detection of drug residues in the tissues of animals.

ACKNOWLEDGMENT

The contribution of Mr. C. DeBoer in the early phase of the assay development is gratefully acknowledged.

SUMMARY

Three types of microbiological assays for porfiromycin were developed:

1. Disc-plate assay with S. aureus FDA-209P on brain-heart infusion agar with a sensitivity of 0.75 μg/ml.
2. Disc-plate assay with S. aureus UC-607 on nutrient agar with a sensitivity of 0.02 μg/ml.
3. Turbidimetric assay with S. aureus UC-607 which has a sensitivity of 0.001 μg/ml.

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DISCUSSION

DR. POLLARD: I hesitated to discuss the first two papers or ask questions on them because of the possibility that this subject might be mentioned in the last two. Doctor DeBoer mentioned the possible cancerolytic effect of this drug on KB-3 and I would like to ask him if he would possibily expand on this. What is the cyto-toxicity level of this drug on normal tissues? Were his experiments performed in tissue culture? How do these results compare with neoplasms in situ?

DR. SAVAGE: The significance of the tissue culture inhibition work, I believe, is of little interest to this group unless it is accompanied by animal studies. A paper is being prepared and will be published soon on the antitumor activities in animals and in tissue culture of this antibiotic, porfiromycin. I believe it will be best if we simply indicate at this time that this antibiotic, like the mitomycins, has antitumor activity.

DR. BURNS: I wonder whether one of the authors might be willing to tell us something of the toxicity of this antibiotic?

DR. SAVAGE: In our early studies, porfiromycin seemed to be of high promise because of its relative nontoxicity. By mitomycin standards in mice, it was some one-eighth to one-twelfth as toxic. Subsequent toxicologic studies, which also will have to be published elsewhere, indicate closer correlation between porfiromycin and the mitomycins in dogs and in monkeys. The data lead us to believe that any approach to the clinic would have to be extremely cautious.

DR. HOUSMAN: I wonder whether anything can be said on the chemical structure of the compound?

DR. HERR: I don't think at this time we can say very much about the chemistry primarily because this work is going on at the moment.

Q: I would like to ask two questions. First, does porfiromycin show any cross resistance to mitomycin? Second, what is the meaning of "more than eight" in the therapeutical trial on the Gram-negative bacteria (Table 5)? Do you mean that you have been able to cure this infection with more than 8 μg or you have not been able to cure the Gram-negative infection?

DR. LEWIS: In answer to your first question, we haven't studied cross resistance with mitomycin C. In answer to your second question, 8 mg/kg was the highest level we could administer subcutaneously and 20 mg/kg was the highest we used orally in mice, so the CD_{50} was higher than these two figures.

DR. SAVAGE: I should like to expand our answer on the cross resistance to mitomycin C. There is no effort on our part to try to side-step this particular question. Any of you in the audience who have attempted to obtain authentic samples of mitomycin C know that it is extremely difficult. Our total procurement up to the time that we were able to carry out these experiments was some 2 mg. Cross resistance studies will be carried out and will be reported as soon as we can obtain sufficient samples.

Q: Was there a cure or not? Dr. Lewis?

DR. LEWIS: Above levels of 8 mg/kg subcutaneously the animals died from toxicity, so they weren't cured.

ANTIBIOTIC PRODUCTION BY PAECILOMYCES PERSICINUS

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INTRODUCTION

A filamentous fungus of soil origin was isolated which was identified as Paecilomyces persicinus according to the description of Nicot [1]. By streak tests and fermentation methods, it was determined that this organism produces a substance active against Gram-negative and Gram-positive bacteria.

The genus Paecilomyces was established by Bainier in 1907 [2] and its close relationship to Penicillium and Aspergillus was clearly recognized. Wilkins and Harris [3] were among the first to demonstrate antibiotic activity by a species of Paecilomyces, in particular, P. burci. More recently, the production of an antifungal antibiotic, variotin, by Paecilomyces varioti Bainier var. antibioticus was reported [4]. To our knowledge, P. persicinus has not been reported previously to produce antibiotics. Thus, further studies of the antibacterial activity of this organism were considered to be highly desirable.

The present paper describes the production, isolation, purification, and microbiological and chemical properties of the antibiotic obtained from P. persicinus. In vitro and in vivo studies are presented.

FERMENTATION

Maintenance of cultures. P. persicinus was maintained on wheat-germ agar slants [5] at 28.5 C for 5–7 days and then stored at 4 C until needed. Bacterial cultures were grown for 24–48 hr on Eugon agar slants (BBL) and then stored at 4 C.

Microbiological assay. Antibiotic activity was assayed by the broth-dilution method. Tubes of Penassay broth (Difco) at pH 7.0 were inoculated with 0.1% (v/v) of a 24-hr culture of Staphylococcus aureus 209 and incubated at 37 C for 18–24 hr. The end point was taken as the tube with highest dilution of antibiotic which caused growth inhibition. One unit of activity was defined as the minimum concentration of antibiotic per milliliter which completely inhibited the growth of S. aureus 209 for 24 hr.

Shake-flask fermentations. Initial fermentations were carried out in 250-ml Erlenmeyer flasks. A suspension of the organism was prepared from a slant culture in 5 ml of sterile water. The suspension was added to a first-stage seed flask containing 50 ml of fermentation medium consisting of cottonseed meal, 1%; corn steep liquor (Staley, No. 165), 3%; glucose, 3%; and calcium carbonate, 0.5%. The medium was sterilized by autoclaving at 121 C for 15 min. The inoc-

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ulated flasks were incubated for 48-72 hr on a rotary shaker operated at 240 cycles/min and describing a 1-in. circle. Each of the several second-stage seed flasks was inoculated with 5 ml of the resultant growth and incubated for 24 hr on the shaker. A 2-ml portion of the growth from the second-stage seed flasks served as the inoculum for each fermentation flask containing 50 ml of medium. Shake-flask fermentations were harvested after 96 hr, at which time the maximum yield of antibiotic, 100 units/ml, was obtained. The pH of the medium rose from an initial value of about 5.4 to a final figure of approximately 8.1.

Pilot plant fermentation. First-stage seed flasks were prepared by inoculating a portion of a well-grown slant into a 500-ml Erlenmeyer flask containing 100 ml of medium. The afore-mentioned medium was modified by the substitution of 1.5% lactose and 1.5% glucose for the 3% glucose. The inoculated flasks were incubated at 25 C for 2 days on a rotary shaker having a 2-in. throw at 280 rpm. A second-stage seed flask was inoculated with a 10% inoculum from the first stage. This flask was incubated in a manner similar to that of the first for 1 day. The contents of the flask were transferred to a 4-liter flask containing 1 liter of medium. After 3 days of incubation at 25 C on a reciprocating shaker, this flask was used to inoculate a 30-liter stainless steel fermenter containing 20 liters of medium.

Agitation was carried out by mechanical stirring at 390 rpm. Sterile air was introduced at a rate of 2.3 ft^3/min. The temperature for various runs was varied from 25 C to 28 C. No significant variations in potencies attained were noted at the different temperatures. The runs varied in duration from 96 to 120 hr. At harvest, broths averaged 87 units/ml.

RECOVERY AND PURIFICATION

Several methods were tested for the recovery of the antibiotic. The activity could be absorbed onto charcoal (Darco G-60) at pH 8.0 and eluted with methanol, but the recovery was only 10%. An alternate method employed a Darco G-60-Celite column with 60% aqueous acetone as the eluent. The most satisfactory procedure for obtaining a clear filtrate from the harvest broth involved the addition with stirring of sufficient acetone to make a 60% acetone solution. After a short settling period, the mixture could be filtered readily. The acetone was removed in vacuo and an aqueous solution was obtained that was suitable for further purification.

Erratic results were obtained when the charcoal-Celite column procedure was scaled up. A preferable procedure was first to absorb pigments and inactive components with 6% (w/v) of Darco G-60-Celite mixture (1:2) at pH 6.3. The activity was not absorbed. Lyophilization of the filtrate yielded solid material of approximately the same potency, 10 units/mg, as in the procedures previously

Table 1. Recovery Study of Paecilomyces Antibiotic

Stage	Units	Recovery of activity, %
Harvest beer	30,000	100
Aqueous solution (after acetone treatment)	30,000	100
Run-through from Darco G-60-Celite column	22,500	75
Lyophilized solid	18,000	60

mentioned but with a 60% yield (Table 1). The further purification of the crude solid was attempted in several ways.

Fractional precipitation from methanol with diethyl ether resulted in an increase in potency to 13 units/mg, but the over-all recovery was low. Attempts to increase the potency by fractional precipitation from anhydrous ethanol with diethyl ether resulted in the inactivation of the antibiotic. The maximum purification was accomplished by chromatographing a sample of the 10-unit/mg material over granular carbon (Darco, 20–40 mesh). 500 ml of an aqueous solution of the antibiotic, 400 units/ml, was applied to a column 100 cm long and 8 cm in diameter containing 500 g of Darco. After washing the column with 4000 ml of water, which removed the bulk of the inert solids, the activity was eluted with 6% aqueous acetone at a flow rate of 2 ml/min. This chromatography was carried out at 4 C. Although some degradation occurred during the chromatography, the recovery of solid material assaying 30 units/mg was approximately 65% (Table 2). Similar results were obtained when a powdered cellulose column was substituted for the granular carbon, although the capacity was lower. In this case, a 70% n-propanol—water solution was used as eluting solvent.

Table 2. Further Purification of Paecilomyces Antibiotic by Chromatography Over 500 g Darco 20-40 Mesh in a 100 × 8 cm Column at 4C

Crude solid	20 g at 10 units/mg
Inert solids removed by washing with 4000 ml water	15.7 g
Lyophilized solids from 6% aqueous acetone elution	4.33 g at 30 units/mg (approx. 65% recovery)

MICROBIOLOGICAL STUDIES

In vitro. The antibacterial spectrum of the antibiotic is given in Table 3. In all tests, 0.1 ml of a 1 : 10 saline dilution of an 18-hr culture of the organism was employed, and the tubes were read after 18–24 hr incubation at 37 C. Inhibition of growth was noted for both Gram-positive and Gram-negative bacteria,

Table 3. Antimicrobial Spectrum of Paecilomyces Antibiotic

Organism	Medium	Inhibitory concentration, units/ml
Escherichia coli	Penassay broth	20
Erysipelothrix rhusiopathiae	Brain-heart broth	4
Hemophilus influenzae	Brain-heart broth	0.06
Klebsiella pneumoniae	Penassay broth	12
Proteus vulgaris	Penassay broth	20
Pseudomonas aeruginosa	Penassay broth	10
Salmonella paratyphi	Brain-heart broth	4
Salmonella typhosa	Brain-heart broth	4
Shigella sonnei	Penassay broth	30
Staphylococcus aureus 209	Penassay broth	1
Staphylococcus aureus 6	Penassay broth	40
Staphylococcus aureus 42B	Penassay broth	40
Staphylococcus aureus 42E	Penassay broth	40
Streptococcus hemolyticus	Penassay broth	3
Streptococcus pyogenes	Brain-heart broth	4

including three strains of penicillin-resistant S. aureus. The resistant strains required 40 times as much antibiotic for inhibition as the sensitive strain. Hemophilus influenzae was particularly sensitive, requiring only 0.06 unit/ml for inhibition. Good inhibitory activity was also noted against species of Salmonella and Streptococcus. Within the range tested, the size of the inoculum did not greatly affect the inhibitory activity of the antibiotic. Serial saline dilutions of the organisms were added to Penassay Broth in the standard assay procedure. The results are shown in Table 4. When 10% human-blood serum was added in the test medium, twice the inhibitory concentration of antibiotic was required for S. aureus 209.

Table 4. The Effect of the Size of the Inoculum upon the Activity of Paecilomyces Antibiotic

Organism	Inoculum, cells/ml	Minimum inhibitory concentration, units/ml
Staphylococcus aureus 209	2.5×10^6	1
	2.5×10^4	1
	2.5×10^2	0.5
Shigella sonnei	9.0×10^6	30
	9.0×10^4	30
	9.0×10^2	10

In vivo studies. The intravenous injection of Paecilomyces antibiotic in doses of 5, 10, and 25 mg (30 units/mg) dissolved in sterile saline was well tolerated by 16- to 20-g white Swiss mice. Upon autopsy, 30 min or 24 hr after injection of these doses, the mice exhibited no macroscopic or histologic abnormalities of the heart, liver, lungs, spleen, intestinal tract, or brain. Injection of 25 mg of antibiotic (30 units/mg) by intraperitoneal and subcutaneous routes was tolerated without evidence of toxicity. Maximum dose applied to mice was therefore 1250 mg/kg, indicating a low order of acute toxicity.

Table 5. Number of Surviving White Swiss Mice Infected Intraperitoneally with 0.3 ml 18-hr-old Culture of Diplococcus pneumoniae Type III ATCC 6303, 10^{-2} Saline Dilution, and Effect of Treatment with Paecilomyces Antibiotic

Hours	Control infected, no treatment	Subcutaneous* administration of antibiotic	Gavage administration† of antibiotic
0	6	6	10
24	6	6	10
36	0	6	5
48	0	6	3
72	0	6	2
96	0	6	1
120	0	6	1

*Subcutaneous injection of 10 mg antibiotic at 0, 6, and 24 hr.
†Gavage administration of 18 mg antibiotic at 0, 6, 24, and 36 hr.

White Swiss mice, infected intraperitoneally with an 18-hr-old virulent broth culture of Diplococcus pneumoniae Type III, ATCC 6303 were protected by Paecilomyces antibiotic administered subcutaneously as well as orally (Table 5).

CD_{100}* for Paecilomyces antibiotic solids was accomplished subcutaneously with 30 mg in mice that received ten times the lethal dose of virulent D. pneumoniae intraperitoneally (10^{-3} broth dilution). CD_{100}* for one times the lethal dose of D. pneumoniae ranged from 7.5 to 15 mg of antibiotic. Protection afforded white Swiss mice by gavage administration of antibiotic was of a lower order, however. In contrast to the control-infected group, which showed 100% mortality in 36 hr, infected gavage-treated animals showed significant protective effects. A similar number of mice that received gavage administration of water at 0, 6, 24, and 36 hr were alive at 120 hr without signs of illness. It was concluded that Paecilomyces antibiotic afforded significant protection by the subcutaneous route against lethal doses of D. pneumoniae, Type III, and that it was absorbed by the oral route in sufficient concentration to afford protection, albeit to a lower degree.

IDENTIFICATION OF THE ANTIBIOTIC

The similarity of the antibacterial spectrum of the Paecilomyces antibiotic to that of penicillin and the subsequent finding that the activity was rapidly destroyed upon treatment with penicillinase, suggested that a penicillin-type antibiotic was being produced. After alkaline hydrolysis at room temperature, 30,000 units of antibiotic absorbed 0.5 meq of iodine [6, 7]. Following acid hydrolysis with 1.0 N hydrochloric acid at 100 C for 7 hr, β-thiovaline was detected by the method of Pal [8]. The antibiotic activity was not extractable from either acidified broth or concentrated aqueous solutions by organic solvents such as ether, amyl acetate, or butanol. This suggested that the antibiotic under test might be a bipolar type of penicillin. Comparative studies were, therefore, carried out with a known bipolar penicillin to establish the identity of the antibiotic. Ascending paper chromatograms of a crude concentrate of the antibiotic in 70% aqueous n-propanol showed (a) one bio-active spot; (b) several ninhydrin-positive areas, the major area coinciding with bio-activity; and (c) a strong carbohydrate spot (detectable with aniline phthalate, alkaline silver nitrate, or resorcinol—hydrochloric acid spray reagents) overlying the bio-active region.

Experiments designed to determine whether the carbohydrate was a component part of the antibiotic molecule ultimately disclosed that the carbohydrate could be separated from the biological activity by paper chromatography in a n-butanol—water—acetic acid (125 : 125 : 30) solvent system. The carbohydrate was the slowest moving component (Rf = 0.11–0.13). The biological activity was associated with the faster moving (Rf = 0.48–0.53) of the two main ninhydrin-positive spots. When a known bipolar penicillin, synnematin B† (cephalosporin N), was chromatographed in this system, two ninhydrin-positive spots were obtained in the identical location of those from the Paecilomyces antibiotic. When the faster moving zones of both the Paecilomyces antibiotic and synnematin B were eluted and re-chromatographed in the same solvent system, some of the slower moving zone (Rf = 0.23–0.25) was again obtained. This indicated that the slower, ninhydrin-positive, biologically inactive zone was produced by the degradation of the antibiotic in this acid system. The carbohydrate was also separable iontophoretically from the biological activity in pyridine acetate at pH 4.0. Biological activity was associated with the ninhydrin-positive zone having the fastest mobility toward the anode, while the carbohydrate was associated with a ninhydrin-positive zone having slight mobility toward the cathode. Synnematin B exhibited identical ninhydrin-positive zones, free of carbohydrate.

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*100% protective dose.

†Synnematin B was supplied by Dr. J. C. Sylvester, Abbott Laboratories, North Chicago, Illinois.

The carbohydrate accompanying the Paecilomyces antibiotic gave a positive Molisch test and it reduced alkaline cupric tartrate. It gave a positive anthrone test and a negative orcinol reaction. A positive Seliwanoff test indicated the possible presence of free or combined ketohexoses or heptuloses. After reacting the carbohydrate with diphenylamine in a mixture of acetic and hydrochloric acids, absorption in the visible range of the spectrum exhibited a maximum at 635 mμ and a minimum at 560 mμ, characteristic for ketohexoses [9]. An osazone was prepared, but it did not possess a definitive crystal structure.

Table 6. The Rf Values of Paecilomyces Antibiotic and Other Penicillins in Various Solvent Systems

Solvent system	Rf value			
	Paecilomyces antibiotic	Synnematin B	Penicillin G	Penicillin O
n-Butanol – acetic acid – water (125 : 125 : 30)	0.48-0.53	0.48-0.53	–	–
Diethyl ether	0.67-0.70	0.68-0.74	0.74	0.24
70% Aqueous n-propanol	0.37-0.41	0.37-0.41	0.50	–

Because of the similarity of the paper chromatographic (Table 6) and iontophoretic behavior of the Paecilomyces antibiotic and synnematin B, a comparison was made of their reactivities toward reagents employed by Newton and Abraham [10]. After treatment with 1.0 N hydrochloric acid for 6 hr, both antibiotic preparations yielded aminoadipic acid, as determined by electrophoresis in pyridine acetate at pH 4.0. Treatment of the antibiotic as well as synnematin B with benzoyl chloride, in sodium acetate buffer (pH 8.0), showed by paper chromatography in 70% aqueous n-propanol that a new bio-active material had formed. These n-benzoyl derivatives possessed identical Rf values. On a paper chromatogram sprayed for the detection of carbohydrate, a single spot was observed with an Rf value unchanged from that of the original material. Finally, treatment of a sample of the Paecilomyces antibiotic and synnematin B with penicillinase and subsequent paper chromatography revealed the loss of bio-activity in both samples. The ninhydrin-positive spots associated with bio-activity disappeared and the ninhydrin-positive spot attributed to the penicilloic-type acid became more intense, with identical Rf values in both samples. A paper chromatogram sprayed for the carbohydrate component showed only a single spot with an Rf value unchanged from that in the penicillinase-untreated material.

It was concluded that the antibiotic produced by P. persicinus is identical with synnematin B. This conclusion was arrived at on the basis of similarity of antibacterial spectrum, inactivation with penicillinase, paper chromatographic and iontophoretic behavior, and chemical studies. This is believed to be the first report of the production of a penicillin-type antibiotic by a species of the genus Paecilomyces.

SUMMARY

A filamentous fungus of soil origin identified as Paecilomyces persicinus was found to produce an antibiotic active against Gram-positive and Gram-negative bacteria. The fermentation medium consisted of cottonseed meal, corn steep liquor, glucose, and calcium carbonate. The highest yields of antibiotic,

100 units/ml, occurred after 96 hr incubation at 28.5 C. Various methods were employed for the recovery and subsequent purification of the active material. The preferred procedure involved treatment of the active filtrate with a charcoal-Celite mixture to remove impurities. Lyophilization of the filtrate yielded solids having a potency of 10 units/mg. The potency of the solids was increased to 30 units/mg by chromatography over granular carbon. The antibiotic preparation thus obtained was relatively nontoxic and protected white Swiss mice infected with *Diplococcus pneumoniae* Type III by both subcutaneous and oral routes. Additional studies employing paper chromatography, iontophoresis, and various chemical procedures indicated the antibiotic to be identical with synnematin B (cephalosporin N). This is believed to be the first report of the production of a penicillin-type antibiotic by a species of the genus Paecilomyces.

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THE PRODUCTION OF SYNNEMATIN B BY PAECILOMYCES PERSICINUS IN A CHEMICALLY DEFINED MEDIUM

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INTRODUCTION

In 1951, Gottshall et al. [1] isolated an antibiotic-producing fungus which Roberts [2] later classified as Cephalosporium salmosynnematum. Olson et al. [3] isolated the active material, identifying two fractions, synnematin A and synnematin B. Simultaneously, Abraham et al. [4] reported the production of cephalosporin N by a species of Cephalosporium originally isolated by Brotzu [5]. The two antibiotics were found to be identical in 1955 [6]. Recently, Grosklags and Swift [7] showed C. salmosynnematum to be a new species of the genus Emericellopsis, E. salmosynnemata, and Kavanagh et al. [8] reported synnematin B production by other members of this genus.

The report of Pisano et al. [9] on the production of synnematin B by Paecilomyces persicinus increases the number of genera containing species known to synthesize this antibiotic. The complex nature of the substrate employed by these investigators for the production of synnematin B (Table 1) made it unsuitable for studies designed to gain insight into the fermentative chemical changes attending the synthesis of synnematin B by P. persicinus. In order to accomplish this objective, the production of synnematin B by P. persicinus in a chemically defined medium was investigated.

Table 1. Composition of the Media Employed

Component	Concentration, g/liter	
	Chemically defined medium*	Complex medium [9]†
Glucose	50	30
Corn steep liquor	–	30
Cottonseed meal	–	10
Betaine	0.50	–
DL-asparagine	9	–
$CaCl_2$	–	5
Na_2SO_4	0.90	–
KCl	0.38	–
$MgCl_2 \cdot 6H_2O$	0.33	–
$NaH_2PO_4 \cdot H_2O$	0.20	–
$FeSO_4 \cdot 7H_2O$	0.025	–
$ZnSO_4 \cdot 7H_2O$	0.020	–
$CuSO_4 \cdot 5H_2O$	0.00032	–

*The chemically defined medium was prepared in distilled water and adjusted to pH 7.0 with NaOH.

†The complex medium was prepared in tap water.

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EXPERIMENTAL

Fermentation. The fermentation studies were conducted according to the procedure of Pisano et al. [9] with minor modifications. The growth from the first-stage seed flask was washed three times with sterile, distilled water. Replicate 250-ml Erlenmeyer flasks containing 50 ml of a chemically defined medium (Table 1) based upon the substrate of Harvey and Olson [10] were inoculated with 2 ml of the washed mycelial suspension. Preliminary trials showed that higher yields of antibiotic were obtained when glucose was substituted for the soluble starch of the original substrate. Consequently, glucose served as the major carbon source throughout this study. A 2-ml portion of the growth from the second-stage seed flasks served as inoculum for each fermentation flask. Each flask also contained 50 ml of the defined medium. Duplicate flasks were removed at 12-hr intervals. The mycelial mass was collected by filtration, washed with water, and dried at 100 C for 12 hr. The fermentation filtrates were restored to volume and stored at −15 C.

Assay. The procedure of Pisano et al. [9] was employed in the present study.

Chemical analytical methods. Total carbohydrates, expressed as glucose, were determined by the modified anthrone procedure of Haas and Fleischman [11]. The Somogyi-Nelson procedure [12] was used to measure reducing substances, expressed as glucose. Total nitrogen was determined by means of a Kjeldahl digestion followed by Nesslerization [13]. The method of Rockstein and Herron [14] was used to measure inorganic phosphorus levels. Inorganic sulfur was assayed by the benzidine procedure [15].

Chromatographic procedures. Carbohydrates were determined by descending paper chromatography in the system employed by Whistler and Hickson [16]. The bioautographic procedure of Olson et al. [3] was used to identify the antibiotics produced in the filtrates.

RESULTS AND DISCUSSION

Synnematin B production, in the defined medium employed, reached a maximum of 10 units/ml after 72 hr incubation at 28.5 C. This amount was one-tenth the yield obtained in the complex medium of Pisano et al. [9]. Antibiotic production in both media is compared in Fig. 1. In the complex medium, antibiotic synthesis commenced after 48 hr incubation, reached a peak at 96 hr, and dropped sharply at 120 hr. In the chemically defined medium, synnematin B was detected after 24 hr with maximum yield occurring at 72 hr. Antibiotic levels decreased

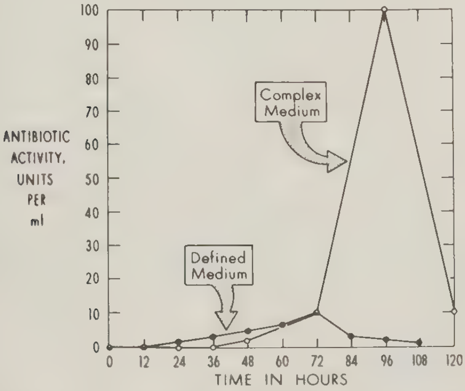

Fig. 1. Synnematin B production by *Paecilomyces persicinus* in two media.

more sharply in the complex medium than in the defined medium, but the yield of synnematin B in the defined medium was considerably lower than in the complex medium. The antibiotic produced in each medium was identified chromatographically by the method of Olson et al. [3].

The growth of P. persicinus as well as the chemical changes attending the synthesis of synnematin B in the chemically defined medium is shown in Table 2. The maximum mycelial yield in the defined medium, 11.2 g/liter, occurred at 96 hr, 24 hr after the highest level of antibiotic was found in the filtrate. Harvey and Olson [10] found that maximum antibiotic production with E. salmosynnemata occurred after the onset of autolysis in their starch-containing medium. Demain [17, 18] reported that penicillin production can be divided into two phases, a phase of rapid mycelial growth followed by a penicillin-production phase. Demain [18] further elaborated on this separation of the growth phase from the antibiotic-production phase, both in chemically defined and in complex media. It was shown that in media containing both glucose and lactose as carbon sources, the glucose is utilized during the growth phase while the lactose is utilized during the antibiotic-production phase. This phase separation is found when either Penicillium notatum or Penicillium chrysogenum strains were employed in the fermentation. As indicated previously, P. persicinus was found to produce synnematin B prior to the occurrence of maximum mycelial yields. In this respect, P. persicinus differs from the Penicillium strains studied by Demain [18] and others [19].

The pH of the synthetic medium employed in this study was adjusted to 7.0 prior to autoclaving. After sterilization it fell to 6.1. The pH dropped to 4.6 within 12 hr after inoculation and then rose to approximately 7 by 24 hr. The pH held at about 7 for the remainder of the fermentation. In the complex medium the pH normally rose to 7.5 within 48 hr and varied between 7.8 and 8.3 at the time of maximum antibiotic production. No antibiotic was detectable in filtrates from the complex medium in which the pH fell below 7.5 after 96 hr of fermentation.

The values obtained for carbohydrate utilization by both the anthrone method for total sugars and the Somogyi-Nelson procedure for reducing substances varied significantly. The possibility of this variation being caused by polymerization of the glucose was investigated by means of paper chromatography. An n-propanol—ethyl acetate—water system, which is employed for the separation of polymeric carbohydrates [16], was used. No evidence for polymerization was found. Haas and Fleischman [11] reported that the anthrone procedure was indifferent to amino acids and peptones, but that pentoses and uronides do react with the reagent. The latter substances, however, could not be detected in the filtrates by chromatographic methods. Amino acids [11, 20] and salts [21] have been shown to interfere in the determination of reducing substances. In the present study, the values obtained by the anthrone procedure were employed for the calculation of the carbohydrate levels in the filtrates. The carbohydrate concentration decreased as a function of the increase in mycelial mass. Ninety-six hours after inoculation, 80% of the carbohydrate had been utilized by P. persicinus. The efficiency of carbohydrate conversion to cell material was found to be 28.7% according to the formula cited by Foster [22]:

$$\text{Efficiency } (\%) = \frac{\text{Cell substance}}{\text{Glucose consumed}} \times 100.$$

The concentration of filtrate nitrogen also decreased as mycelial mass increased. P. persicinus utilized 82.5% of the total filtrate nitrogen after 96 hr.

Table 2. Chemical Changes Attending the Synthesis of Synnematin B by *Paecilomyces persicinus* in a Chemically Defined Medium

Fermentation time, hr	Mycelial yield, g/liter	pH	Synnematin B activity, units/ml	Total sugars as glucose, g/100 ml	Reducing substances as glucose, g/100 ml	Nitrogen, g/100 ml	Phosphate, mg/100 ml	Benzidine-precipitable material as sulfate, g/100 ml
0	1.96	6.07	0	4.95	4.86	0.20	14.00	0.24
12	1.36	4.67	0	4.70	4.17	0.19	8.00	0.23
24	3.96	6.39	2	4.35	3.60	0.17	5.50	0.21
36	5.34	6.95	3	3.30	2.98	0.15	4.00	0.17
48	7.22	6.97	5	2.66	2.32	0.13	3.30	0.16
60	8.94	7.05	7	1.92	1.54	0.10	2.30	0.15
72	9.50	7.05	10	1.53	0.91	0.08	1.70	0.14
84	10.10	7.06	3	1.10	0.55	0.05	0.90	0.14
96	11.28	6.90	2	1.01	0.30	0.04	0.90	0.14

The degree of utilization of asparagine and betaine as nitrogen sources was not determined. This aspect of nitrogen metabolism is now under investigation. The effect of other amino acids, as well as that of various inorganic nitrogen sources, on the production of synnematin B by *P. persicinus* would be of interest since little is known regarding the nitrogen metabolism of this organism.

Phosphorus uptake was not commensurate with cell synthesis. Within the first 12 hr, approximately 43% of the inorganic phosphorus was taken up, whereas cell synthesis was negligible during the same period. The rate of phosphorus uptake decreased with fermentation time, and during the second 12-hr period, only an additional 18% was utilized. Thus, within 12 hr the rate of phosphorus uptake decreased by 58%. After 84 hr incubation, approximately 94% of the inorganic phosphorus had been removed from the medium. No further uptake of phosphorus was noted beyond this period of incubation. It is possible that *P. persicinus* possesses a mechanism for phosphate storage similar to that found in *Cephalosporium longisporum* [23]. Such storage mechanisms have been recognized in other organisms. Resting cells of *E. salmosynnemata* do not appear to have such a phosphate reserve mechanism [24], as evidenced by a significantly decreased ability to oxidize glucose in the absence of added inorganic phosphorus.

A study of the rate of sulfur utilization was attempted. The presence of phosphates and amino acids has been reported to interfere with sulfate values obtained with the benzidine procedure [25]. The values reported in this paper are for benzidine-precipitable material calculated as sulfur. It can be seen from Table 2 that the maximum rate of sulfur uptake occurred after 36 hr followed by a cessation of sulfur utilization after 72 hr. Although the sum of all material precipitated by benzidine is being reported, a comparison of the uptake curves for benzidine-precipitable material, phosphorus, and nitrogen indicates that the inflection points on the benzidine-precipitable-material curve are a function of the sulfur concentration and reflect sulfur uptake from the medium. The physiological significance of this type of uptake curve in *P. persicinus* demands further study, since a direct correlation of the sulfur uptake with either mycelial yield or synnematin B production is not apparent. The maximum rate of uptake of the sulfur complex occurred after 36 hr incubation, while maximum mycelial yield was obtained 96 hr after inoculation, and maximum synnematin B production was noted after 72 hr. Considerable interest has been evidenced recently in the sulfur metabolism of organisms which produce penicillin-type antibiotics. Segel and Johnson [25] found that the high-penicillin-yielding strain, *P. chrysogenum* 51-20F3, was capable of storing high concentrations of ionic inorganic sulfate intracellularly. Although one is unable to draw valid conclusions regarding any possible mechanisms operative in sulfur uptake by *P. persicinus* on the basis of data presented, further study of intracellular sulfur could indicate possible mechanisms.

A comparison of synnematin B production by *P. persicinus* and by *E. salmosynnemata* suggests significant differences in their mechanisms of antibiotic synthesis. Further studies, including those indicated previously, are now under way in an effort to delineate some of the fundamental variations employed by both organisms in the synthesis of synnematin B.

SUMMARY

The production of synnematin B by *Paecilomyces persicinus* was studied in a chemically defined medium. Maximum antibiotic production, 10 units/ml, was obtained after 72 hr incubation, whereas the highest mycelial yield, 11.2 g/liter,

occurred at 96 hr. Approximately 80% of the carbohydrate and 82% of the nitrogen was used during the fermentation. The pH of the medium dropped during the first 12 hr of fermentation from 6.1 to 4.6 and then rose to approximately 7, where it held during the remainder of the fermentation. Of the 94% of the total phosphorus taken up by P. persicinus, almost half was utilized within the first 12 hr after inoculation. The maximum rate of sulfur uptake occurred at 36 hr, followed by a cessation of sulfur utilization after 72 hr.

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DISCUSSION

Q: I would like to know whether there is any evidence that this mold is producing a small amount of cephalosporin C in addition to cephalosporin N.

DR. PISANO: We have no such evidence, primarily because we didn't check for the production of cephalosporin C.

EXPERIENCE WITH A NEW SULFONAMIDE IN THE TREATMENT OF STREPTOCOCCAL PHARYNGITIS

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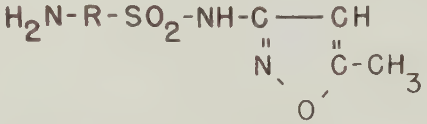
INTRODUCTION

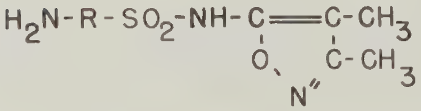
Morris and others [1-5] have indicated that sulfonamides used in the treatment of acute streptococcal pharyngitis, although reducing the incidence of suppurative complications, did not eradicate the streptococci from the oropharynx. It has also been shown that the nonsuppurative complications of streptococcal infections, rheumatic fever and acute glomerulonephritis, are not prevented unless the organism is eradicated [5]. Therefore, it has been recommended that sulfonamides not be used in the treatment of acute streptococcal infections [1, 5-7] although sulfadiazine is recommended for the prophylaxis of rheumatic fever [7-10].

Since the publication of the above studies, new "long-acting" sulfonamides have been developed. It is the purpose of this present study to evaluate the effectiveness of a new sulfonamide, 5-methyl-3-sulfanilamido-isoxazole*, in eradicating the streptoci from the oropharynx (Fig. 1).



5-methyl-3-sulfanilamido-isoxazole

Gantanol



Gantrisin

Fig. 1.

MATERIALS AND METHODS

Ninety consecutive patients with bacteriologically proven beta-hemolytic streptococcal pharyngitis, who were seen at the University of Oklahoma Medical Center Health Service between March and October, 1960, are the subjects of this study. They included 19 males and 71 females ranging in age from 17 to 62 years, with 75% between 17 and 30 years of age (Table 1). All patients were seen for symptoms of acute pharyngitis except two who were found to have these organisms on routine culture. None of these patients were hospitalized. Cultures of the throat were taken with sterile cotton-tipped swabs which were rubbed thoroughly over the posterior pharyngeal wall and the tonsils or tonsillar fossae. The inocula were cultured on blood-agar medium. The organisms were identified by grossly examining the culture growth for beta-hemolyzing colonies and by staining by Gram's method. Grouping and typing of the beta-hemolytic streptococci were not available.

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*Supplied by Hoffman-LaRoche, Inc., as Gantanol.

Table 1. Age Distribution of Patients

Age, in years	Number of patients
Under 20	22
21-30	45
31-40	9
41-50	11
51-60	2
Over 60	1
Total	90

All patients received the same dosage schedule of the drug, 2 g initially followed by 1 g every 12 hr for a total of 10 days. Treatment was begun on the average of 3 days after the onset of symptoms, with a range of 1-7 days. Subsequent throat cultures were taken about 15 days after initiation of therapy, with a range of 11-25 days. This corresponded to culturing 5 days after termination of treatment. Pre- and posttherapy urinalyses and peripheral blood studies were performed in 50% and 28% of the patients, respectively, and no significant abnormalities attributable to the drug were noted.

RESULTS

Of the 90 patients treated, two had minor drug reactions and did not receive full courses of treatment. Of the remaining 88 patients, the post-therapy cultures were negative for beta-hemolytic streptococci in 83 or 94% (Table 2). In five of these 83 successfully treated patients it was necessary to extend the usual treatment period 7 days to achieve bacteriologic conversion. Five patients, or 6%, were treatment failures having positive post-therapy cultures, and one of these five continued to have a positive culture following 10 days treatment with penicillin. One patient had a relapse 11 days after termination of treatment, had a positive culture, and was subsequently treated successfully with penicillin. One patient

Table 2. Result of Treatment:
90 Patients

Cures	83
10-day course	78
17-day course	5
Failures	5
Drug reactions	2
Total	90

also became symptomatic and had a positive culture, but this was four months after the initial treatment and, therefore, the patient was considered to be reinfected.

One-fourth of our patients had follow-up oropharyngeal cultures at intervals of up to twelve weeks after termination of treatment. No additional positive cultures were found.

Patient acceptance of the drug was good, with only two of the 90 patients noting any side reactions; one noted a questionable urticaria and one a macular erythematous rash.

DISCUSSION

Morris and others [1-5] have shown that 1-6 days after termination of sulfonamide treatment in acute streptococcal infections, oropharyngeal cultures are positive for streptococci in 60 to 95% of the patients and 52-95% of the untreated controls, indicating that the sulfonamides used did not eradicate the streptococci. This also indicates that if the streptococci were inhibited during the active phase of treatment, there was a rapid and early regrowth of the organism. In the present study oropharyngeal cultures made on the average of 5 days after termination of treatment were positive in only 6% of the patients, and only one relapse was known to have occurred. To date no known cases of rheumatic fever or acute glomerulonephritis have occurred in this group. These results also compare favorably with those reported with the use of various antibiotics in the treatment of acute streptococcal infections, including those for bacteriological conversion rates: 48-95% with erythromycin [11, 12] 55-76% with tetracyclines [13-15], and 82-99% with penicillin [3-5, 11, 12, 15] (Table 3).

Table 3. Bacteriological Conversion Rates

Agent	Conversion, %
Untreated controls	5-48
Sulfadiazine	5-40
Gantrisin	22
Tetracyclines	55-76
Erythromycin	48-95
Penicillin	82-99
Gantanol	94

The high percentage of bacteriological conversion reported here as compared to that with other sulfonamides may in part be explained by the longer treatment period used here in contrast to the usual 5-day treatment period quoted in most other studies. However, this alone is not a satisfactory explanation because if the growth of streptococci were only inhibited one might expect a greater number of relapses or positive cultures or both than was found in this study.

We realize that grouping and typing of the streptococci isolated in these patients would have been desirable for a more definitive analysis of our results. However, as noted by Wannamaker [16] and Brumfitt [17], at least 65% of these isolates could be predicted to fall into Lancefield group A.

We believe that continued follow-up of our patients is necessary to attempt to estimate the frequency of either acute rheumatic fever or acute glomerulonephritis as well as the recurrence rate of streptococcal carriers in this group.

In our hands this agent has proved to be a safe and highly effective treatment in acute streptococcal pharyngitis, justifying further clinical evaluation under controlled conditions.

ACKNOWLEDGMENT

We are grateful to Mrs. Janice Brower, Mrs. Erma Jean Johnson, Mrs. Marie Dennison, and Mrs. Johnnie Tarpley for their technical assistance.

SUMMARY

Eighty-eight patients with acute beta-hemolytic streptococcal pharyngitis were treated with a new, "long-acting" sulfonamide, 5-methyl-3-sulfanilamido-isoxazole, with a 94% posttherapy bacteriological conversion rate which compares favorably with other therapeutic regimens. In our hands this drug has proven to be a safe, highly effective treatment in acute streptococcal pharyngitis, justifying further clinical evaluation under controlled conditions.

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DISCUSSION

DR. FISHER: What was the comparative rate of clinical response to this sulfonamide as compared to penicillin, for example? Was it slower, equal to, or faster?

DR. BRADEN: It seemed to be equal to that of penicillin in this group of patients. We did not control this with penicillin, which we are planning to do, but my idea of it would be that it would be just as good.

A PRELIMINARY REPORT ON THE LONG-TERM TREATMENT OF CHRONIC PYELONEPHRITIS WITH A NEW SULFONAMIDE*

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INTRODUCTION

In most studies concerned with the treatment of chronic pyelonephritis the incidence of apparent bacteriological cure varies from 10 to 58% [1, 2, 4] depending on the criteria for cure and the number of follow-up cultures. Many studies were based on single cultures taken before, immediately after, and at one to six months following treatment. Thus the comparison of any method of treatment is made difficult and, regardless of the method, treatment still remains unsatisfactory. Because of the latter, the present study was undertaken using a new agent and a thorough follow-up period.

METHODS

Thirty-three patients (27 females and 6 males) were treated with a new long-acting sulfonamide, 5-methyl-3-sulfanilamido-isoxazole.* The similarity between this agent and Gantrisin is seen in Fig. 1.

The distribution of this group (Fig. 2) as to age and sex is similar to that noted in most other studies, that is, females predominate in the child-bearing age group while thereafter incidence is divided evenly between the sexes. For acceptance into the study each patient was required to have a past history of two or more acute exacerbations of pyelonephritis, characteristic physical findings, abnormal urine findings, and a positive urine culture. These patients

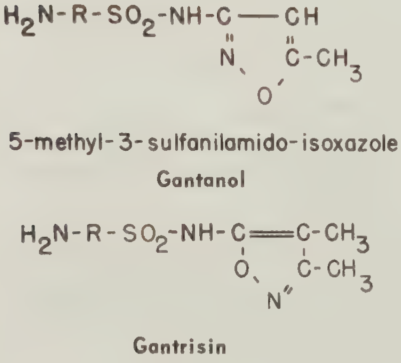

Fig. 1

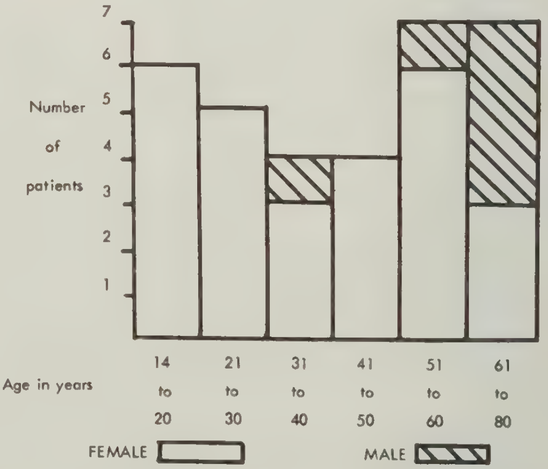

Fig. 2

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*Supplied by Hoffman-La Roche, Inc., as Gantanol.

have been followed for two to six months and have been seen at biweekly or monthly intervals. At each visit all patients were seen by one of us and had a urinalysis, hemogram, and quantitative urine culture. Urines were clean-catch midstream collections and were cultured by the pour-plate method. During treatment each patient had an intravenous pyelogram, and intermittently they had liver function studies, blood urea-nitrogen determinations, and PSP and urea clearances. All patients were then placed on 5-methyl-3-sulfanilamido-isoxazole, 1 g twice daily after an initial priming dose of 2 g. Our predetermined course of therapy was set at three months.

Table 1. Original Organisms in Urine Cultures

Organism	Number of cultures	Percent of total
Escherichia coli	15	43
Nonhemolytic Staphylococcus	9	26
coagulase-positive (6)		
coagulase-negative (3)		
Aerobacter aerogenes	4	11
Proteus mirabilis	4	11
Pseudomonas aeruginosa	3	9
	35	100

RESULTS

The organisms recovered on initial urine culture are shown in Table 1. In two instances the original urine culture was a mixed infection; hence there were 35 organisms on original isolation in 33 patients. The colony counts on these cultures are shown in Table 2. Thus, in 90% of the cultures there were more than 10^5 colonies/ml of urine, which is in accord with the probability curve noted by Kass [3]. Three of the four patients with less than 10^5 colonies/ml initially, subsequently had greater than 10^5 colonies, and the other demonstrated the same organism consistently on repeated cultures.

Table 2. Colony Counts of Original Urine Cultures

Cultures	Count/ml
25	10^6
6	10^5
3	10^4
1	10^3

After initiation of treatment it was a common occurrence to find different organisms on subsequent cultures. Thirty-seven new organisms were recovered on reculture and Table 3 demonstrates the distribution of these organisms. In 12 instances the organisms were obtained in pure culture and in 12 instances they were obtained in mixed culture. Eleven of the 12 mixed cultures contained two organisms, and one had three organisms. Of particular interest was the presence of coagulase-negative staphylococci in 14 instances. These organisms were included only if in a concentration of greater than 10^5 colonies/ml. In one of these instances the same organism was also cultured from a percutaneous renal biopsy. We, in accordance with the generally accepted view, will regard these as contaminants.

Table 3. New Organisms Cultured During Treatment

Classification	Number
Nonhemolytic Staphylococcus	15
coagulase-positive (1)	
coagulase-negative (14)	
Hemolytic Streptococcus	5
Aerobacter *aerogenes*	5
Escherichia coli	5
Proteus mirabilis	4
Pseudomonas aeruginosa	3
	37

Of the 33 patients only five would satisfy our strict criteria for bacteriological "cure," i.e., three successive sterile urine cultures. If we altered these criteria to two successive sterile cultures the incidence of "cures" would rise to 11 of 33, and if we required only a single sterile culture the incidence would increase to 24 of 33. Thus, the incidence of "cure" varies inversely with the number of sterile urine cultures.

Of the five patients who would satisfy our strict criteria for bacteriologic "cure," all remain asymptomatic and have continued sterile urine cultures, though they are each now two months past treatment. If we then approach the problem from the point of view of how often the initially cultured organisms were eradicated, we find that of the initial 35 organisms five, including one Proteus and one Aerobacter, were eradicated with treatment.

In vitro disc sensitivities of 5-methyl-3-sulfanilamido-isoxazole against cultured bacteria were performed on 29 occasions. Fourteen of these 29 bacteria were insensitive to the sulfonamide. In five instances this resistance was noted prior to patient contact with the drug, and these additional patients were not placed on the drug. In the remaining nine cases resistance appeared after the patient had been treated with the sulfonamide.

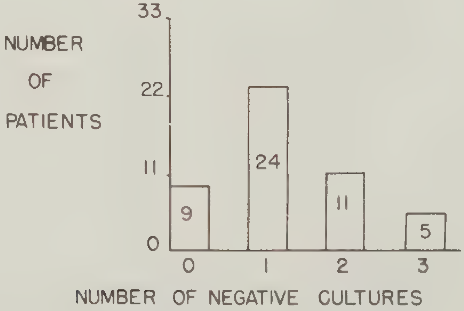

Fig. 3

The drug was clinically well accepted by 32 of the 33 patients. One patient developed a red macular rash three days after the inception of therapy. The rash disappeared after voluntary discontinuance of the drug. This patient subsequently admitted to a known sulfonamide sensitivity. Of the 15 patients who had liver function studies performed, one had a transient elevation of transaminase which subsided promptly with discontinuance of the sulfonamide.

Case R.C. is a 71-year-old Caucasian male with chronic pyelonephritis and bladder calculi. He had a transurethral resection in October, 1959, and at that time received two units of blood. When seen by us seven months later he complained of anorexia and tiredness. He was diagnosed as having chronic pyelonephritis, and Pseudomonas aeruginosa was isolated from the urine in a concentration of 10^6 colonies/ml. The patient was placed on 5-methyl-3-sulfanilamido-isoxazole and one week later had elevation of SGOT to 180 and SGPT to 140. Because of this finding and the lack of response of the organism to therapy, the drug was discontinued and the transaminase returned to normal.

There were no other evidences of toxicity to this agent in any of the parameters studied.

As is shown in Table 4, the sulfonamide has been discontinued in 22 patients, in ten cases because of completion of the arbitrary three months of treatment. This latter group includes the five instances of bacteriological "cure." In 12 patients treatment was discontinued because of lack of response in five, toxicity in two, death in two, and three were lost to follow-up. The cause of death in the two patients was lymphocytic leukemia in one and a cerebral vascular accident in the other. Eleven patients are still receiving the sulfonamide.

Table 4. Reasons for Discontinuance of Sulfonamide

Reason	Cases	"Cures"
Completed treatment	10	5
Lack of response	5	0
Lost to follow-up	3	0
Toxicity	2	0
Death	2	0
	22	5
Still on treatment	11	
	33	

SUMMARY

We treated 33 patients with chronic pyelonephritis with a new long-acting sulfonamide. Only five of the 33 had a bacteriological "cure" by our strict criteria. Our experience, and that of other investigators treating similar patients with a variety of chemotherapeutic agents, suggests that this agent is as successful as any other single agent.

We believe further that though our incidence of "cure" is low it is because of the nature of the disease and because of our careful follow-up studies. In terms of toxicity this sulfonamide was well accepted by 32 of 33 patients. The only toxic reactions noted were a transient elevation of transaminase in one patient and a mild dermatitis in another.

CONCLUSION

5-methyl-3-sulfanilamido-isoxazole is relatively a safe chemotherapeutic agent, its major advantage being the decreased frequency of administration. Except for this it appears no more potent than other sulfonamides. The percentage of apparent "cures" varies inversely with the thoroughness of the bacteriological follow-up. In the great majority of patients the treatment of chronic pyelonephritis is as yet unsatisfactory in terms of permanent sterilization of the urine.

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CLINICAL EFFECTS OF MIKAMYCIN OINTMENT ON INFECTIOUS SKIN DISEASES

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INTRODUCTION

We present here the therapeutic results of treatment of various infectious skin diseases with a new antibiotic, mikamycin.

Mikamycin was applied topically in the form of vaseline-base ointments in concentrations of 2, 1, and 0.5%. The effects were remarkable in the treatment of superficial infectious skin diseases. Clinically, mikamycin ointment was characterized by its low irritability.

In addition it is of interest that mikamycin was very effective against Staphylococcus aureus cultured from skin lesions (Table 1) with a low minimum inhibitory concentration as compared to those of other antibiotics (Table 2).

Table 1. Sensitivity Test with Various Kinds of Antibiotics*

Causative organism	Resistance examination							
	Sulfa	PC	SM	CM	TC	EM	KM	MM
Staphylococcus aureus	−	−	+	++	++	+++	(++)	++
"	−	−	++	++	++	++	+	++
"	−	+	+	++	++	+++	++	++
"	−	−	+	++	++	++	(+)	++
"	−	+	+	++	++	+++	++	+
"	+	−	(++)	++	++	+++	++	++
"	−	+	+	+	++	++	+	++
"	++	+	++	++	+	++	++	++
"	−	−	+	++	+	++	+	++
"	−	−	++	++	+++	++	++	++
"	−	−	(+)	(+)	+	++	(+)	++
"	−	−	+	+	++	++	+	++
Streptococcus	−	+	−	++	+	+++	−	−

*PC, penicillin; SM, streptomycin; CM, Chloromycetin; TC, tetracycline; EM, erythromycin; KM, kanamycin; MM, mikamycin.

Parentheses indicate resistance of other strains to antibiotic

RESULTS

Mikamycin ointment was applied two or three times a day for as long as two weeks but most of the cases responded well in four to five days. The therapeutic results are shown in Table 3.

In the present study of 190 cases, mikamycin ointment was remarkably effective in 112, effective in 34, ineffective in 44, and the over-all effective rate

Table 2. Minimum Inhibitory Concentration of Each Antibiotic for Staphylococcus aureus

Antibiotic	Number of cultures inhibited				
	>100 μg/ml	100 μg/ml	10 μg/ml	1 μg/ml	0.1 μg/ml
MM	2	1	0	22	5
I	4	1	0	18	7
CM	1	2	25	2	0
KM	1	1	16	12	0
TC	3	1	19	7	0
SM	5	0	12	12	1

Table 3. Effectiveness of Mikamycin Ointment Against Various Skin Diseases

Diseases	Number of cases	Response		
		Excellent	Good	Poor
Impetigo contagiosa	76	58	11	7
Eczema impetiginosum	17	7	6	4
Secondary infections	24	17	4	3
Pustular miliaria	4	2	1	1
Furuncle and furunculosis	24	11	3	10
Folliculitis	13	7	2	4
Pyoderma	5	2	1	2
Acne	18	3	4	11
Ulcer	5	2	1	2
Burns	4	3	1	0
Total	190	112	34	44

was 77.0%. However, the rate in superficial infections, including impetigo, impetiginous eczema, secondary infections, pustular miliaria, and burns was 84.8%, better than that for treatment of deep pyoderma, including furuncle, furunculosis, folliculitis, acne, and similar infections. This may be because bacteriostasis occurs only on the superficial skin lesions with such treatment. It is our opinion that combined local and oral administration of antibiotics forms the best treatment of deep pyoderma. We could not find any remarkable difference in the response to mikamycin ointment among the 2, 1, and 0.5% drug concentration levels.

The above-mentioned findings lead us to conclude that mikamycin ointment is a new weapon against superficial skin infections. The fact that mikamycin has a strong bacteriostatic effect on staphylococci cultured from the lesions, even if the bacteria are resistant to other antibiotics, may support our conclusion.

A FEW BASIC OBSERVATIONS ON MIKAMYCIN, A NEW ANTIMICROBIAL ANTIBIOTIC, AND ITS CLINICAL TRIALS
PRELIMINARY REPORT

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ORIGIN AND CHEMICAL STRUCTURES OF MIKAMYCIN

Mikamycin, a new antimicrobial antibiotic, is produced in a culture broth of a strain of Streptomyces which was isolated from a soil sample obtained in the suburbs of Tokyo and was named Streptomyces mitakaensis by Umezawa et al.

There are two fractions of mikamycin, known as mikamycin A and mikamycin B. The former is a yellowish crystalline substance having a bitter taste and melting at 178 C with decomposition. The chemical structure of mikamycin A is still unknown, but Umezawa et al. have reported recently that mikamycin A is degraded by mild alkaline hydrolysis in 0.5 N NaOH at room temperature into two fragments: mikamycinine, $C_{22}H_{32}O_7N_2$, and mikaic acid, $C_7H_9O_3N$. Okabe [9] is now approaching an understanding of the complete structure of mikamycin A. Mikamycin A is freely soluble in chloroform, methanol, ethanol, and acetone; soluble in methylene chloride, ethylene chloride, benzene, and ethyl acetate; slightly soluble in ether and water; and insoluble in petroleum ether, carbon tetrachloride, and hexane.

Mikamycin B is a colorless, crystalline substance and decomposes at 260-265 C. The formula $C_{45}H_{54}N_8O_{10}$ was established by elementary analysis and molecular weight determination. Hydrolysis, isolation of each amino acid, and C-terminal amino acid determination of partially hydrolyzed products have revealed that mikamycin B is composed of 3-hydroxypicolinic acid, L-threonine, D-α-amino-n-butyric acid, L-proline, L-p-dimethylamino-N-methylphenylalanine, L-4-oxopipecolic acid, and L-phenylglycine. It is freely soluble in acetone, butyl acetate, benzene, and chloroform; soluble in methanol and ethanol; and insoluble in water, petroleum ether, and n-hexane. However, mikamycins A and B are soluble in distilled water at 21 C to the extent of 500 μg/ml and 150 μg/ml, respectively.

ANTIBACTERIAL ACTIVITIES OF MIKAMYCIN

Antimicrobial activities of mikamycins A and B were first determined by Umezawa et al., by the dilution method, revealing marked sensitivities of Gram-positive organisms and Hemophilus pertussis. The exceptions are a few strains of hemolytic streptococci. It is interesting that remarkable synergistic activities between these two mikamycins have been confirmed. The mixtures (ratios from 90% A and 10% B to 10% A and 90% B) exhibit marked synergism (Table 1).

Moreover, it has been proved by in vitro test that the mikamycins show no cross resistance to other available antibiotics and sulfa drugs such as penicillin,

Table 1. Comparison of In Vitro Activities of Mikamycin

Test organism	Minimum inhibitory concentration, μg/ml		
	Crude* powder	Mikamycin A	Mikamycin B
Staphylococcus aureus 209P	0.4	2.0	10.0
Sarcina lutea	0.1	0.2	2.0
Streptococcus pyogenes gr. A type 19 (J17D)	0.4	4.0	4.0
Streptococcus pyogenes gr. A type 3 (B930/24)	0.4	4.0	4.0
Diplococcus pneumoniae type I	1.0	16.0	1.0
Diplococcus pneumoniae type II	1.0	16.0	2.0
Diplococcus pneumoniae type III	1.0	16.0	1.0
Hemophilus pertussis	0.2	0.4	10.0
Brucella melitensis	10.0	20.0	10.0

*The crude powder of mikamycin mixture contains 84% A and 16% B.

streptomycin, chloramphenicol, tetracycline, and others, although mikamycin B exhibits less activity against erythromycin- and magnamycin-resistant staphylococci. Kishimoto and others reported lower sensitivity of kanamycin-resistant staphylococci against a mikamycin mixture. The staphylococci are among the most sensitive of mikamycin-susceptible pathogenic bacteria. Forty-eight of 50 coagulase-positive Staphylococcus strains, isolated at the Kanto Teishin Hospital, Tokyo, were inhibited by 0.312 μg/ml or less of mikamycin mixture; only two strains (4%) required 0.625 μg/ml of the drug for inhibition. Yamanaka reported that 28 of 31 (96.8%) staphylococcal strains derived from clinical sources at the Department of Surgery, Osaka City University Medical School were sensitive to 1.25 μg/ml or less of mikamycin mixture.

TOXICITIES OF MIKAMYCIN

Yamanaka reported acute intraperitoneal toxicity of mikamycin mixture (85% A and 15% B) to be low, and its LD_{50} was 860-930 mg/kg. Daily intraperitoneal injections of 350 mg of the mixture (80-90% A and 20-10% B) for 14 days in mice did not cause any toxic signs. Ohkoshi [10] gave dogs 100 mg/kg of oral mikamycin mixture daily for 2 months and did not observe vomiting, diarrhea, or any other side effects. Laboratory examinations of the blood, liver, and kidneys of the dogs indicated nothing significant. Observations of Yamanaka on seven dogs which had been given 1 g of oral mikamycin mixture every 2 days for a period of three months revealed the same findings.

BLOOD LEVELS AND URINARY EXCRETION OF MIKAMYCIN

A method of bio-assay of mikamycins in body fluids was devised by Watanabe et al. [17] using the synergistic relationship between mikamycins A and B. Yamanaka studied blood levels of the drug in dogs first and showed that oral administration of 2 g of mikamycin mixture produced 1.0-1.4 μg/ml of A and 0.15

μg/ml of B in blood serum 1-2 hr later. Merely 0.3-0.4 μg/ml of A and no demonstrable B was found in the blood 1-2 hr after oral intake of 1 g of the mixture.

Oral administration of 1 g of the mixture in capsules (70% A and 30% B) to 17 healthy adult males of about 60 kg body weight showed no demonstrable mikamycins in the blood plasma. A few cases showed low blood levels of A and no evidence of B after oral intake of 1.25 g or more of the mikamycin mixture. Generally speaking, it may be said that the peak blood level is reached 3 hr after oral administration of 1.25-1.5 g of mikamycin mixture with a resultant blood concentration of 0.15 μg/ml. A blood level of 0.3 μg/ml develops 4.5 hr after oral intake of 2.5 g of the mixture. No cases were experienced showing demonstrable mikamycins in the cerebrospinal fluid after oral intake of the drug. Yamanaka points out that body fluids such as defibrinated blood, erythrocyte suspension corresponding to 1% hematocrit, or hemolyzed blood solutions treated with either distilled water or saponin inactivate mikamycin A. Every milliliter of normal blood inactivates about 0.1 mg of mikamycin mixture. However, the modes of inactivation of mikamycins by the body fluids are still unknown and are to be found by further investigations.

Significant urinary excretion of mikamycins was noticed; 0.99 mg A and 0.5 mg B were recovered in the urine within 3 hr after oral intake of 1 g of the mixture and 2.2 mg A and 0.9 mg B after 2 g intake. It is clear that 0.3-0.4% of the mikamycins are recovered in the urine 7.5 hr after oral administration.

CLINICAL TRIALS OF MIKAMYCIN

Since April, 1960, a committee has been organized for clinical trials of mikamycin in Japan. Topical use of mikamycin ointment for infectious skin lesions will be discussed by other coworkers at this conference, and we present here the results of clinical trials of oral mikamycins which consisted of 85% A and 15% B (250-mg mixture in a capsule).

Mikamycin in urology. Some infectious diseases in the urological field were first treated with oral mikamycin by Professor Ohmura and his coworkers (Department of Urology, Okayama University Medical School) and Hino et al. (Department of Urology, Kyoto University Medical School), all of whom were members of the committee.

Ohmura et al., prescribed oral mikamycins, 1-2 g a day for 3-5 days, for 12 cases of cystitis, 11 of which had originated after surgery on the urinary bladder, urethra, prostate, or penis for either cancer or stone. The infections were caused by staphylococci, hemolytic streptococci, Escherichia coli, or mixed cultures. Following 2- to 3-day doses of mikamycins, disappearance of staphylococci and streptococci from the urine was noticed, along with improvement in the microscopic findings and prompt subsiding of fever in 3 out of 5 cases. However, E. coli in the urine showed no response to oral mikamycins although both cystitis and urethrocystitis caused by E. coli showed clinical responses evidenced by improvement of other urinary findings and of subjective complaints of the patients in 5 out of 7 cases. One case of acute hemorrhagic cystitis did not respond to oral mikamycins. However, there was one case of nonspecific urethritis caused by Staphylococcus albus that disappeared dramatically following oral mikamycin administration in a dose of 2 g/day (12 g in total). Another patient with acute epididymitis was placed on 2 g of mikamycins daily for 5 days, resulting in alleviation of pains and reduction of swelling.

Hino and his coworkers gave 1 g of oral mikamycins daily to 17 patients in the urological clinic. Three out of 4 cases of acute cystitis caused by Staphylococcus aureus responded effectively, being improved following a total oral dose of 4-6 g. One case of acute cystitis and 5 cases of chronic cystitis revealing staphylococci and E. coli in the urine were placed on oral mikamycins combined with either tetracycline or sulfa drugs, and three of them recovered within 6-8 days. There were 2 cases of acute pyelitis, one caused by S. aureus and the other by both staphylococci and E. coli. The former was treated with oral mikamycins only and the latter with mikamycins combined with sulfa drugs, and both of them showed subsiding of fever within 3-5 days and complete recovery within 4-7 days. However, only one out of 6 cases of nongonococcal urethritis caused by either staphylococci or streptococci manifested a bacteriological cure. Four patients were free of subjective complaints immediately after oral intake of mikamycins and the remaining two did not show any signs of improvement. One case of acute gonococcal urethritis responded excellently to a total of 3 g of mikamycins, revealing negative gonococci in the urine and alleviation of subjective symptoms.

Untoward side effects were rarely seen with oral mikamycins. When seen, they were evidenced by anorexia and pain in the epigastrium in one out of 15 cases treated by Ohmura and one out of 17 cases treated by Hino.

Mikamycin therapy has been effective on staphylococcal cystitis and pyelitis, and on gonococcal urethritis, although its bacteriological effectiveness on nongonococcal urethritis was not always guaranteed. Mikamycins alone were not effective in mixed infections with E. coli, which seem to require combined therapy with other kinds of antibiotics active against Gram-negative pathogenic bacteria.

Mikamycin in internal medicine. Professor Torii (Department of Internal Medicine, Hokkaido University Medical School) and Professor Kawamori et al. (Department of Internal Medicine, Kumamoto University Medical School) have just started to try mikamycins clinically in the field of internal medicine.

Torii treated the following 11 cases with 1 g/day oral administration of mikamycins: pulmonary abscess, 3; bronchiectasis, 1; chronic bronchitis, 1; acute pneumonia, 1; cholecystitis complicated with pancreatitis, 1; tonsillitis, 2; acute cystitis, 1; and pyelitis, 1. All the pulmonary cases except acute pneumonia raised sputum with Gram-positive bacteria which became nonpurulent and decreased in volume following 5-13 days of treatment. A case of acute pneumonia responded excellently to 8 g of mikamycins, x-ray films showing disappearance of shadow in the lower field of the left lung. A case of chronic cholecystitis complicated with pancreatitis also responded well to 7 g of oral mikamycins, resulting in negative Gram-positive bacteria in the bile and relief of pains. Of the 11 cases, one developed diarrhea and one a skin rash as slight side effects of oral mikamycins.

Kawamori and his co-workers gave 1-1.5 g of mikamycins daily to 2 cases of bronchiectasis, and to one case each of pulmonary abscess and meningitis. Clinical effectiveness of oral mikamycins has been not sufficiently confirmed by these authors.

Professor Donomae and his coworkers (Department of Internal Medicine, Osaka University Medical School) reported a case of pulmonary abscess which raised sputum containing Diplococcus pneumoniae. The bacterium was resistant to penicillin, chloramphenicol, and streptomycin originally and became resistant to tetracycline and sulfa drugs after their use. This case, which was placed on 1 g of mikamycins daily for 4 days, showed subsidance of fever, decreased volume of sputum, and remarkable improvement in x-ray pictures. These authors also observed clinical and bacteriological improvements with mikamycins in a few cases of pulmonary abscess complicated with bronchiectasis and revealing streptococci and staphylococci resistant to penicillin and sulfa drugs in the sputum, and in tonsillitis complicated with prostatitis caused by staphylococci and streptococci resistant to tetracycline, penicillin and streptomycin.

Clinical trials of oral mikamycins in the field of internal medicine have recently started, but not enough cases have been accumulated to decide the final effect of the drug. The drug was effective in 14 out of 18 cases, most of which had been suffering from infectious pulmonary diseases caused by Gram-positive bacteria resistant to all of the other antibiotics available at present.

Mikamycin in general surgery. Mikamycins were also tried clinically at the Department of Surgery, Osaka City University Medical School and the Department of Surgery, the Kanto Teishin Hospital.

Mikamycin mixture in capsules was administered orally 1-2 g/day q.i.d. for adult patients. Some of the patients with open wounds were treated by topical use of mikamycin suspension in a saline solution.

The results of clinical trials of oral mikamycins in the field of general surgery are tabulated in Table 2.

Table 2. Results of Clinical Trials of Mikamycins in General Surgery

Clinical diagnosis	Ishiyama et al.			Shiraha et al.		
	Number of cases	Effective	Ineffective	Number of cases	Effective	Ineffective
Furuncle	6	2	4	6	6	
Carbuncle	1	1		2	2	
Panaritium	3	3		6	6	
Abscess	3	3		2	2	
Infected wound	3	3		3	2	1
Mastitis	1	1		3	3	
Tonsillitis	2	2				
Phlegmon	1	1		3	3	
Urinary infection	3	2	1	1	1	
Osteomyelitis				2	2	
Erysipelas				2	1	1
Acute epididymitis				1		1
Rheumatoid arthritis				1		1
Pyothorax				1	1	
Total	23	18 (78.3%)	5 (21.4%)	33	29 (87.8%)	4 (12.1%)

Ishiyama and his coworkers prescribed the mikamycin mixture for 23 cases of infectious deep skin and tissue lesions of which 18 cases (78.3%) responded excellently. Twenty-nine (87.8%) out of Shiraha's series of 33 cases consisted of similar surgical lesions which responded effectively to the oral mikamycin mixture. Many of these cases simultaneously underwent minor surgery. There were 31 strains of staphylococci isolated from the lesions of Shiraha's series and the results of their sensitivity tests are listed in Table 3. These surgical staphylococcal strains showed resistance to one or more antibiotics and sulfa drugs, but all of them were sensitive to mikamycins, 3 strains being inhibited by 2.5 μg/ml and 28 strains (96.8%) by 1.25 μg/ml or less.

Among the 33 cases of Shiraha's series there was one case each of anorexia, diarrhea, and gastric pains and 2 cases of vomiting as untoward side effects caused by oral mikamycins. However, meticulous examinations revealed no changes in blood findings on liver or kidney function during and after mikamycin therapy.

Table 3. Antibiotic Sensitivity of Infecting Staphylococci

Case number	Clinical diagnosis	Antibiotic sensitivity*							Inhibited by mikamycins, µg/ml
		SF	PC	SM	TC	CM	EM	OL	
1	Cystitis	S	S	S	R	R	S	S	0.31
2	Furuncle	S	R	S	S	S	S	S	0.62
3	Furuncle	R	R	S	R	R	S	R	0.62
4	Periproctal abscess	R	S	R	R	S	S	S	0.62
5	Furuncle	R	S	S	S	S	S	S	0.62
6	Infected wound	R	R	R	R	S	S	S	0.62
7	Otitis media	R	R	S	R	S	S	S	0.62
8	Phlegmon	R	S	R	R	S	S	S	0.62
9	Mastitis	R	R	R	R	S	S	S	0.62
10	Periproctal abscess	R	S	R	R	S	S	S	0.62
11	Infected wound	R	R	S	R	S	S	S	0.62
12	Furunculosis	R	S	S	S	S	S	S	0.62
13	Undecided (pus)	R	S	S	S	S	S	S	0.62
14	Panaritium	R	R	S	S	S	R	S	0.62
15	Panaritium	R	S	S	R	S	S	S	0.62
16	Abscess	S	R	R	R	S	S	S	1.25
17	Furuncle	S	R	S	R	S	S	S	1.25
18	Panaritium	S	R	R	R	S	S	S	1.25
19	Mastitis	R	R	S	R	S	S	S	1.25
20	Furuncle	S	R	S	S	S	S	S	1.25
21	Carbuncle	R	R	S	S	S	S	S	1.25
22	Furuncle	R	R	S	R	S	S	S	1.25
23	Infected wound	R	S	S	S	S	S	S	1.25
24	Infected wound	R	R	R	R	S	S	S	1.25
25	Furuncle	R	S	S	S	S	S	S	1.25
26	Infected wound	R	S	R	S	S	S	S	1.25
27	Panaritium	R	S	S	R	S	S	S	1.25
28	Furuncle	R	S	R	S	S	S	S	1.25
29	Infected wound	R	S	S	R	S	S	S	2.5
30	Thrombophlebitis	R	R	R	R	S	S	S	2.5
31	Gas gangrene	R	R	S	S	R	R	R	2.5

*SF, sulfa drug; PC, penicillin; SM, streptomycin; TC, tetracycline; CM, chloramphenicol; EM, erythromycin; OL, oleandomycin; R, resistant; S, sensitive.

ACKNOWLEDGMENT

The authors wish to express their sincere thanks and appreciation to Professor Hamao Umezawa, M.D., Institute for Applied Microbiology, Tokyo, for his timely suggestions and kind help.

Mikamycins used in present clinical trials were kindly supplied by Kanegafuchi Chemical Industry Co. Ltd., Osaka, Japan.

SUMMARY

Mikamycin is a new antimicrobial antibiotic consisting of two fractions which show remarkable antibacterial synergism, and possessing strong bacteriostatic activity against Gram-positive bacteria, particularly on pathogenic staphylococci resistant to known antibiotics.

The authors, members of a committee for clinical trials of mikamycin in Japan, have just started to try the antibiotic clinically in the field of general surgery and the results obtained are presented in this paper together with the preliminary reports of coworkers in the fields of urology and internal medicine.

Many of the urological infections caused by staphylococci responded excellently to oral mikamycins as evidenced by bacteriological and clinical improvements. A few cases of nongonococcal urethritis, however, remained unchanged following mikamycin therapy, and mixed infections, particularly with *E. coli*, required combined treatment with mikamycins and antibiotics effective against Gram-negative bacteria.

Experiences with mikamycins in the field of internal medicine are still poor although a few infectious diseases in the bronchopulmonary system have been treated with dramatic and excellent responses as evidenced by improvements in clinical symptoms and signs, x-ray films, and bacteriological findings.

Clinical effectiveness of mikamycins has also been confirmed in the field of general surgery, revealing a 78.3-87.8% rate of cure, comparable with other antibiotics available at present.

It is noteworthy, also, that mikamycins are effective against surgical staphylococci resistant to other antibiotics and exhibit no cross resistance. Untoward side effects caused by oral mikamycins have been rarely experienced. Side effects are manifested by such minor symptoms as anorexia, vomiting, diarrhea, and rash, without any disturbances in the blood findings or functions of parencymatous organs.

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DISCUSSION

DR. BEHRENS (Lilly): In defining clinical usefulness, it is certainly very desirable that the infecting organism be determined so that the antibiotic can be used most wisely. I wonder whether the speaker has any information regarding the infecting organism in these clinical cases?

DR. SAKAI: All patients were studied bacteriologically and many pathogenic organisms were isolated.

DR. EHRLICH: I'm afraid, Dr. Behrens, that a more detailed statement is not possible this morning. It's obvious, though, that the majority were staphylococci. I see by the manuscript that there were 31 strains of staphylococci. What types they were and what other species were involved are not stated.

DR. HILLEGAS (Parke Davis): Although the results seem to show synergistic effect between the two mikamycins, the apparent absorption of mikamycin B seems to be rather small. I wonder whether the author have tried just mikamycin A.

DR. SAKAI: We have never tried just mikamycin A. We have only used the mikamycin complex A and B.

DR. HILLEGAS: Then following up on Dr. Behrens' question, I wonder if perhaps Dr. Sakai could answer by telling us what the failures were due to.

DR. SAKAI: What do you mean, failures?

DR. HILLEGAS: You had success, I believe, in 88% of your cases, and I wonder what the reason was for failure in the remaining 12%.

DR. EHRLICH: Perhaps we might put the question this way, Doctor. Were the failures due to insensitive organisms or to anatomic inaccessibility of the organism, or have you any other explanation for the failures? Was it insensitivity?

DR. SAKAI: No.

DR. EHRLICH: They were all sensitive. The answer as I get it is that sensitivity tests were performed. The organisms were all sensitive and the reason for the 12-13% failures is not clear.

GEMINIMYCIN, A NEW SYNERGISTIC ANTIBIOTIC

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The word synergism in biological systems has meant different things to different people. Webster's Dictionary [1] defines it as "the cooperative action of discrete agencies such that the total effect is greater than the sum of the two effects taken independently." On the other hand, Gould's Medical Dictionary [2] while agreeing with the above also defines it as "the harmonious and cooperative action of two or more agents or organs, as two muscles cooperating to produce a movement which neither alone could effect."

Examples of the first type have been the most common and are seen in the cases of several drugs. A recent example of such in the antibiotic field is the combination of antibiotics PA 114A and PA 114B [3]. A unique example of the second type has now been found in which neither of the components alone shows any measurable activity while a combination of the two produces activity. This antibiotic complex is termed geminimycin and its isolation and characterization is the subject of this paper.

The culture, which is an unidentified streptomycete, is grown submerged in the usual complex natural media. The progress of the fermentation is followed by activity against *Bacillus subtilis* or *Micrococcus pyogenes* var. *aureus*. At 28-30 C the antibiotic activity reaches a maximum in about 40-50 hr and falls off rapidly after this period.

Preliminary efforts to isolate the active material by the usual solvent extraction, adsorption, or precipitation techniques led to complete loss of activity, even under mild conditions. This loss of activity was gradually traced to a step which involved dialysis at 5 C. Even though there was no indication of instability under these conditions, no activity was found in either the dialyzable or nondialyzable fractions. Combination of the two portions restored the original ac-

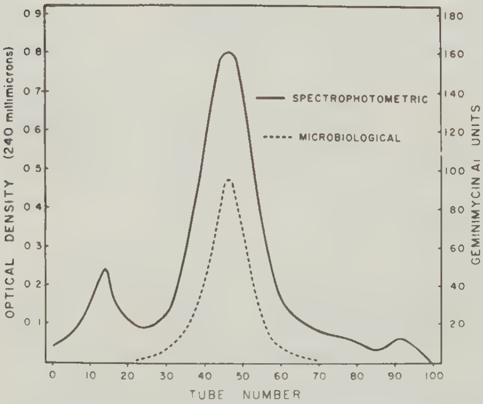

Fig. 1. Countercurrent distribution I.

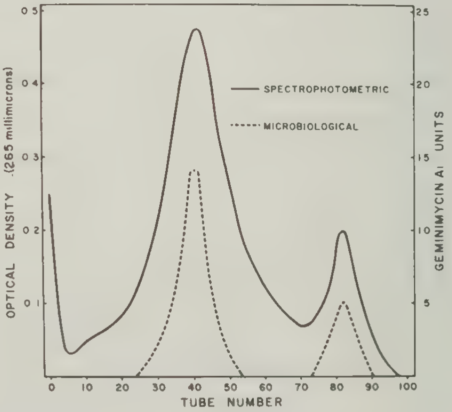

Fig. 2. Countercurrent distribution II.

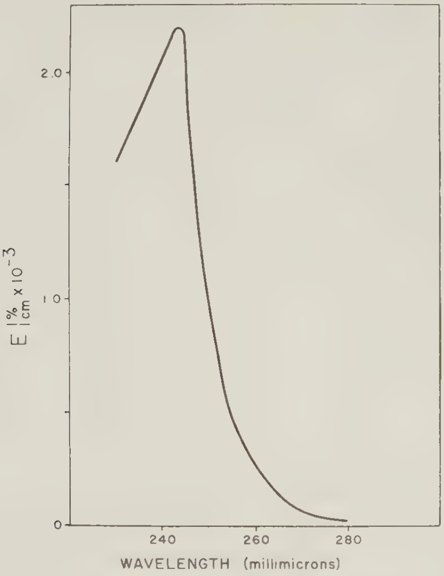

Fig. 3. Ultraviolet spectrum of geminimycin A-1.

tivity. The dialyzable fraction was termed geminimycin A and the nondialyzable fraction geminimycin B.

The fractions obtained during the isolation of geminimycin A are assayed by the following method. The samples are plated on B. subtilis plates, alone as well as after mixing with an equal weight of geminimycin B. The purity of the fractions is indicated by their lack of activity when plated alone.

ISOLATION

For the isolation of geminimycin A the broth is concentrated and dialyzed. The dialyzable fraction is reconcentrated and extracted with n-butanol. After removal of the solvent the product is subjected to countercurrent distribution in a system composed of ethyl acetate, isopropyl ether, methanol, and water mixed in equal proportions. The progress of the distribution is checked by following microbiological activity as well as optical density at 245 mμ. The distribution pattern is shown in Fig. 1. The major active portion from the distribution was recovered and subjected to another countercurrent distribution in a chloroform—

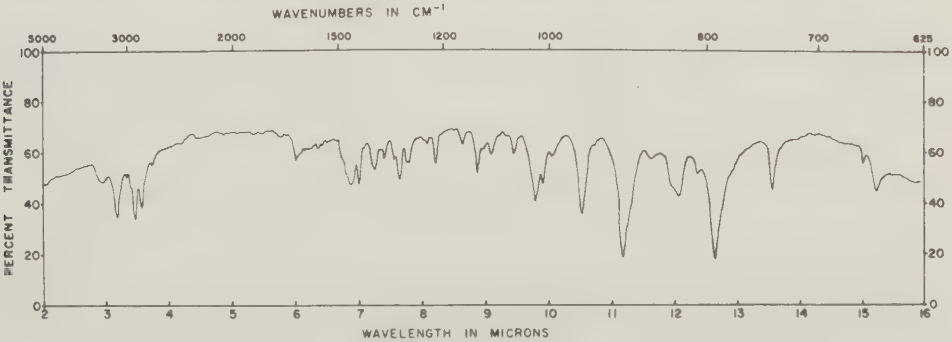

Fig. 4. Infrared spectrum of geminimycin A-1.

Table 1. Synergism of Dialyzed Geminimycin Broth

Dialysate (geminimycin A), % by weight	Nondialyzable (geminimycin B), % by weight	*Bacillus subtilis* zone, mm
100	0	none
90	10	23.5
80	20	25.1
70	30	24.1
60	40	23.3
50	50	22.0
30	70	19.6
10	90	15.2
0	100	none
66.3[*]	33.7[*]	22.5

[*] Approximate broth composition.

ligroin—methanol—water system (7 : 3 : 7.5 : 2.5). Figure 2 shows the resolution of geminimycin A into components A-1 and A-2, the former being the major one. Geminimycin A-1 is crystallized from isopropyl ether and finally sublimed at room temperature under 0.2-0.5 mm pressure, whereby it is obtained as colorless long needles which melt at 56-57 C. Analysis yielded the following percentages: C, 72.24; H, 8.02; N, 8.49. Figures 3 and 4 show the ultraviolet and infrared spectra of geminimycin A-1. The substance is very unstable to storage even at 5 C, the crystals gradually changing to a brownish resin.

Geminimycin B was not obtained in pure form from the nondialyzable portion although some of the characteristics were obtained by utilizing the synergistic activity. This activating power is destroyed in solution below pH 6 and above pH 8 and also by heating at 60 C at pH 7 for 10 min. By the use of ammonium sulfate

Table 2. Synergism of Geminimycin A-1 and Geminimycin B Against *Bacillus subtilis*

Geminimycin A-1 concentration, μg/ml	Zone of inhibition, mm	Geminimycin B[*] concentration, μg/ml	Zone of inhibition, mm
1000	None	10,000	None
200[†]	32.2	500[§]	31.0
100[†]	29.9	250[§]	29.4
50[†]	26.9	125[§]	24.2
25[†]	25.6	62.5[§]	22.5
12.5[†]	22.7	31.25[§]	19.5
6.25[‡]	19.2	500[‖]	25.6
3.125[‡]	18.6	250[‖]	25.6
1.5625[‡]	15.7	125[‖]	23.5
		62.5	20.5
		31.25	17.7

[*] Lyophilized preparation from nondialyzable portion of filtered broth.
[†] Plated in combination with 500 μg/ml of geminimycin B.
[‡] Plated in combination with 50 μg/ml of geminimycin B.
[§] Plated in combination with 200 μg/ml of geminimycin A-1.
[‖] Plated in combination with 50 μg/ml of geminimycin A-1.

precipitation the activity is found to be present in the precipitate at 50% saturation. It is also found to be precipitated in the cold by acetone or ethanol at 90% concentration.

Table 3. Synergism of Purified Geminimycins

Bacteria	Minimum inhibitory concentration, μg/ml		
	Geminimycin A-1	Geminimycin B	Equal wt mixture
Staphylococcus aureus	>1000	>1000	31.5
Streptococcus pyogenes	>1000	>1000	1.9
Escherichia coli	>1000	>1000	15.7
Salmonella typhosa	>1000	>1000	7.8
Klebsiella pneumoniae	>1000	>1000	3.9

SYNERGISTIC ACTIVITY

Table 1 shows the activity of the combination of the two components in the crude form. It appears that the ratio of the two components as present in the broth is not necessarily the ideal one for maximum activity. Table 2 shows the activity of pure geminimycin A-1 with varying levels of crude geminimycin B and vice versa against *B. subtilis*. The effect of maintaining the concentration of one component and varying that of the other is seen clearly from this table. Synergistic activity against other microorganisms is shown in Table 3. Alone, neither of the components, A or B, of geminimycin was toxic to mice (5-day i.p. test) at 100 mg/kg, but a combination of the two (1:1 by weight) was toxic at less than 50 mg/kg.

ACKNOWLEDGMENTS

The authors are grateful to Dr. A. R. English for supplying the data in Table 3 and to Dr. J. J. Oleson for the toxicity studies.

SUMMARY

Isolation of geminimycin, which exhibits a unique example of synergism in the antibiotic field, has been described. This antibiotic consists of a dialyzable component (geminimycin A) and a nondialyzable component (geminimycin B) neither of which is active alone but exhibit activity in combination.

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DISCUSSION

DR. KATAGIRI (Sloan-Kettering): Has the antitumor activity of this substance been tested?

DR. RAO: It is primarily active on microorganisms; it has very mild, sometimes questionable activity on tumors. We don't think it has much chance to be an antimicrobial agent, much less an antitumor agent. Geminimycin B from all the properties looks like a macromolecular compound. Because of lack of interest we didn't pursue this component.

DR. DONOVICK (Squibb): What is the structure of these compounds and the nature of their toxicity?

DR. RAO: I'm sorry to say that neither of these is available. We know the composition of the compound and some of the properties, but not the structure, and the toxicity was the gross toxicity.

DR. HOOGERHEIDE (Delft): I was struck by the fact that this is not what I would call real synergism. We have been talking about synergism quite a bit and usually in the sense of just a little better. But here you have a clear cut case where actually neither of the two substances is active and the combination is. I wonder, therefore, whether here there isn't something else behind it. Couldn't it be something like an association or a chemical combination between the two that you pull apart by dialysis?. Is there anything known about this, about the combination between the two substances?

DR. RAO: The only information that could be of use in that line is the ultraviolet spectrum of the two components. There is some change when the two are mixed. Of course we are not on very firm ground when we say this because geminimycin B is anything but pure. Whether that slight change in the spectrum denotes some kind of combination between the two components is not known. Also, I want to point out in answer to the two previous questions that synergism, according to most dictionaries, actually means an increase of activity better than the algebraic sum of the two. Whether it could be little more or much more, it is still defined that way. But this may be a more unique synergism, where there is no activity for either of the components singly — something reminiscent of an enzyme-coenzyme relationship.

ISOLATION AND CHARACTERISTICS OF FRENOLICIN

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A new antibiotic named frenolicin was obtained in crystalline form from culture filtrates of a soil isolate identified as Streptomyces fradiae [1].

The isolation of this antibiotic was accomplished by solvent extraction, chromatography on magnesia-silica gel, and crystallization from benzene. The analytical data suggest the empirical formula $C_{13}H_{14}O_5$.

This paper presents chemical and biological characteristics of this antibiotic.

EXPERIMENTAL

Fermentation and assay. Frenolicin was produced by cultivation of S. fradiae (Lederle No. AC-860) under conventional agitation and aeration conditions on a medium with the following composition: soybean meal, 0.5%; glucose, 1.5%; molasses distiller's dried solubles, 0.5%; glycerol, 0.75%; NaCl, 0.5%; $MgSO_4 \cdot 7H_2O$, 0.1%; $CaCO_3$, 0.5%; tap water.

The in vitro antibiotic activity was assayed by the agar-diffusion method utilizing Bacillus subtilis ATCC 6633 grown on Difco nutrient agar at pH 6.0. Purified preparations were diluted in 40% aqueous methanol for assay.

Frenolicin activity of various preparations was also followed by paper chromatography and bio-autography with the above assay organism and medium. With a developing solvent consisting of dimethylaniline saturated with 0.02 N ammonium hydroxide, frenolicin had an R_f of 0.07. With the upper phase obtained by mixing o-xylene, cyclohexane, glacial acetic acid and water (100 : 100 : 25 : 50), an R_f of 0.85 was observed.

Isolation and chemical properties. The process for the isolation of crystalline frenolicin was as follows. Forty-eight-hour fermentation broths of S. fradiae were filtered with filter aid. The filtrate was adjusted to pH 4.0 with concentrated HCl and then extracted with a two-thirds volume of ethyl acetate. The ethyl acetate extract was concentrated in vacuo to an oil. To this oily residue were added 10 volumes of ethanol—benzene (1 : 99), and the filtered solution was chromatographed on a column packed with acid-washed magnesia-silica gel (Florisil). The column was developed with the ethanol-benzene solution until the first yellow band containing frenolicin was collected as a fraction. This solution was concentrated to dryness in vacuo. The residue was extracted with hot benzene. On cooling of the benzene solution to room temperature, frenolicin crystallized. The antibiotic was recrystallized several times from benzene. Yields were 50-75 mg of crystals per liter of culture filtrate.

Frenolicin crystallized as pale yellow needles, mp 160–161 C. The antibiotic is readily soluble in methanol, ethanol, ethyl acetate, acetone, glacial acetic acid, and carbon tetrachloride; somewhat less soluble in benzene; and essentially insoluble in water, cyclohexane, and petroleum ether. It dissolves with decomposition in aqueous alkali but it is stable under acid and neutral conditions. Aqueous $KMnO_4$ is slowly decolorized by frenolicin but there is no uptake of bromine in carbon tetrachloride or glacial acetic acid. There is no reaction of frenolicin with nitrous acid, p-nitrobenzenediazonium-fluoroborate, or 2,4-dinitrophenylhydrazine, but with $FeCl_3$ a brown-red color results.

Analyses calculated for $C_{13}H_{14}O_5$ are C, 62.3; H, 5.6; for one $-CCH_3$ per molecule, 6.0; mol wt, 250. Found: C, 62.7; H, 5.72; no halogen, N, or S; $-CCH_3$, 3.84; mol wt (Rast-Exaltone) = 228. Titration data indicated pK'_{a_1} 6.2 (equiv wt = 246); pK'_{a_2} = 10.0 (equiv wt = 124). $[\alpha]_D^{25} = -37.7°$, c = 1.564 (MeOH). Hydrogen uptake (PtO_2 in ethanol) was 1.99 moles H_2 per mole. The ultraviolet and infrared spectra of the antibiotic are presented in Figs. 1 and 2, respectively. In methanol, ultraviolet absorption maxima were at 234 and 363 mμ with $E_{1\,cm}^{1\%}$ = 520 and 155, respectively.

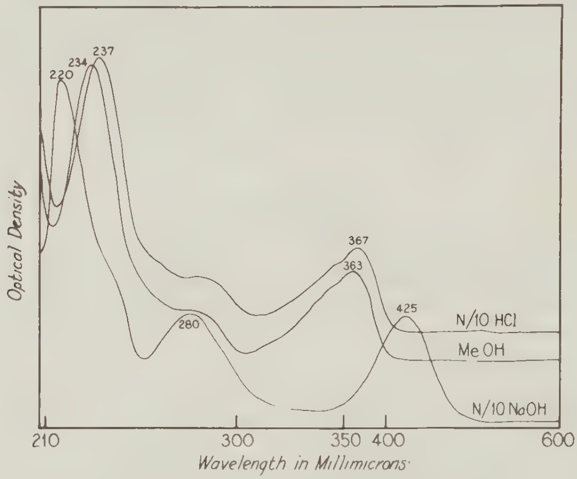

Fig. 1. Ultraviolet absorption spectrum of frenolicin.

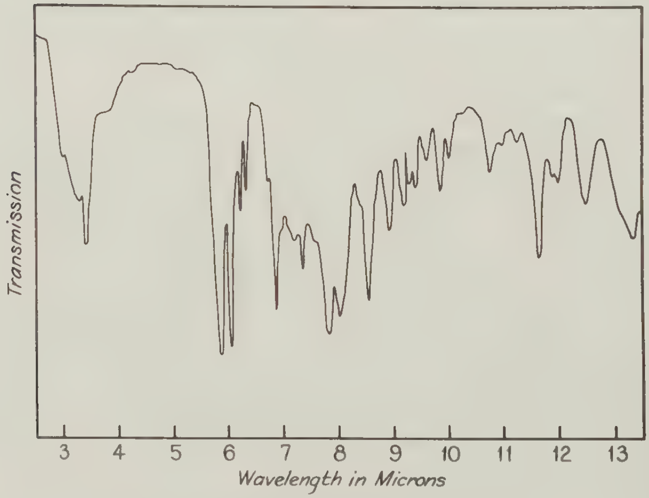

Fig. 2. Infrared spectrum of frenolicin.

These data differentiate frenolicin from previously announced antibiotics. The $-CCH_3$ analysis suggests one group per molecule. The pK'_{a_1} and infrared bands at 3.3, 3.8, 5.85 μ suggest the presence of a carboxyl group. The pK'_{a_2}, infrared bands at 7.85 and 8.05 μ, and shift of the UV absorption maxima to higher wavelengths by addition of alkali are suggestive of the presence of a phenol or enol grouping. Hydrogenation studies indicate the presence of two readily reducible bonds per molecule.

Biological properties. The antimicrobial spectra of frenolicin are shown in Tables 1 and 2.

Table 1. Antibacterial Activity of Frenolicin*

Organism	Minimal inhibitory concentration, μg/ml
Mycobacterium ranae	125
Mycobacterium smegmatis ATCC 607	>250
Staphylococcus aureus ATCC 6538P	31
Streptococcus faecalis ATCC 8043	125
Bacillus subtilis ATCC 6633	15
Streptococcus pyogenes C2O3	31
Streptococcus sp. nonhemolytic No. 11	125
Staphylococcus albus No. 69	31
Streptococcus sp. β-hemolytic No. 80	125
Staphylococcus aureus NY 104	62
Bacillus cereus ATCC 10702	31
Pseudomonas aeruginosa ATCC 10145	>250
Proteus vulgaris ATCC 9484	>250
Escherichia coli ATCC 9637	>250
Salmonella gallinarum	>250
Escherichia coli No. 22	>250

*Agar dilution technique on asparagine dextrose agar.

Table 2. Antifungal Activity of Frenolicin*

Organism	Minimal inhibitory concentration, μg/ml
Candida albicans Bergen strain E-3	>250
Saccharomyces cerivisiae	>250
Mucor ramannianus M-143	>250
Fusarium episphaeria F-105	250
Homodendrum cladosporoides Z-516	250
Trichophyton mentagrophytes E-11	125
Candida mycoderma	>250
Penicillium digitatum P-308B	62
Myrothecium verrucaria Z-131	250
Alternaria fasciculata Z-625	250
Aspergillus fumigatus S-246	>250

*Agar dilution technique on trypticase soy agar.

Frenolicin was inactive in protection tests when administered subcutaneously at 40 mg/kg to mice infected with *Staphylococcus aureus* Smith, *Streptococcus pyogenes* C-203, or *Diplococcus pneumoniae* SV1. In other in vivo tests no activity was observed with PR8 No. 18 and SK viruses; sarcoma 180, lymphosarcoma 6C3HED, and mammary adenocarcinoma tumors; Nematospiroides, Oxyurids, and Hymenolepis parasitic infestations.

ACKNOWLEDGMENTS

The authors wish to acknowledge the cooperation of the following personnel in our laboratories: L. Brancone, A. C. Dornbush, W. Fulmor, J. H. Martin, J. N. Porter, P. Shu, and D. E. Smith and associates for microbiological and chemical assistance. For in vivo test results, we are indebted to E. H. Dearborn, E. Waletzky, H. J. White, and their associates.

SUMMARY

Frenolicin, an antibiotic produced by a strain of Streptomyces fradiae, has been isolated in crystalline form. Analyses correspond to an empirical formula $C_{13}H_{14}O_5$. Physical and chemical data indicate the presence of enolic (phenolic) and carboxyl groups and two unsaturated bonds.

This antibiotic has a moderate degree of activity against Gram-positive bacteria, a low order of activity against selected fungi, and no activity against Gram-negative bacteria. No in vivo protection activity was observed.

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DISCUSSION

DR. EHRLICH: What was the toxicity in terms of LD_{50}?

DR. VAN METER: I can't give you that. The data are not available.

DR. FUSARI (Parke-Davis): What catalyst was used to obtain the hydrogenation value? Your slide indicates a 2-mole uptake of hydrogen, whereas a phenolic structure such as you proposed would be expected to take up 3 moles.

DR. VAN METER: Platinum dioxide in ethanol.

A NEW FORM OF STABILIZED PEROXIDE AS A CHEMOTHERAPEUTIC AGENT

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INTRODUCTION

The impact of antibiotics on the practice of medicine has brought with it several problems. Outstanding, of course, is the serious problem of antibiotic-resistant strains of organisms such as Neisseria gonorrhoea and especially Staphylococcus aureus. There is also the great increase in the occurrence of moniliasis following prolonged antibiotic therapy, especially with penicillin.

Preoccupation with the steadily expanding use of antibiotics and with the development of new types and new combinations has also had another effect, a tendency to neglect other antimicrobial agents, including some which had proved efficacious in the past, which are free from the limitations of the antibiotics, and which often possess unique and special qualities of their own. It is for this reason that for some time we have been interested in the utilization of hydrogen peroxide and more particularly in some of its later improved forms, which we believe can find an important place in medicine.

Previous papers by one of the authors [1] have referred mainly to one new form of hydrogen peroxide, a solution of urea peroxide in a special anhydrous glycerine vehicle. The present paper presents some preliminary studies of a related product in a different dosage form, that of an ointment. This form seems to offer some new and interesting possibilities. Because of its density, the ointment has obvious advantages over the liquid form in many topical applications. Perhaps of greater interest is the fact that it seems to have unusual cleansing power for infected and contaminated lesions. Finally, there are indications that it is effective against some, if not all, resistant strains of Staphylococcus aureus.

A brief review of the history of hydrogen peroxide as an antibacterial agent seems in order.

For a great many years only the 3% aqueous solution of hydrogen peroxide was utilized. Eventually it was established that however effective this product might be in vitro against a wide range of organisms, its rapid enzymatic breakdown in contact with most body fluids rendered it of very limited value in vivo. This general appraisal is expressed by Carrel et al. [2] as follows:

> "Hydrogen peroxide solution gives encouraging results when its bactericidal action is examined in a test tube. But on wounds on the contrary it has a very feeble action because it decomposes with the greatest readiness under the influence of the catalysis always going on in the tissues and in the blood corpuscles. Consequently its action is only exerted during a comparatively insignificant period of time. The mechanical detergent action which results from the rapid disengagement of oxygen when in contact with infected surfaces, has probably a greater value than the antiseptic action of the hydrogen peroxide itself."

The desirable qualities of hydrogen peroxide, its cleansing action and freedom from toxicity or sensitizing action, led to studies to determine if hydrogen peroxide could be made more effective in vivo. These later studies mainly involved the utilization of other peroxides in other vehicles. The work of Meleney [3] in utilizing zinc peroxide, the efforts of Reid et al. [4] to prepare an ointment utilizing various peroxides dispersed in glycol polyesters, and the work of Brown [5] and his associates with solutions of urea peroxide in polyhydric alcohols are examples of this continuing interest in peroxide as an antibacterial agent. Urea peroxide in glycerol solution has been reported favorably for its value in dental therapy [1] and for the treatment of various forms of pharyngitis [6]. One of the present authors has also evaluated this product by a novel technique in which the bactericidal action is measured in the presence of thin blood films [7]. This work gives support to the clinical observation that the slower breakdown of this solution does, in effect, give it appreciable antimicrobial action in contact with body fluids, thus overcoming in some measure the limitation of the aqueous hydrogen peroxide.

The success of urea peroxide in glycerol solution stimulated further work which resulted in an ointment-like preparation with similar properties. This preparation was designated for experimental work as T-3*. The ointment-like form offers obvious advantages in many topical applications where an ointment is more practical than a liquid. In addition it aims at utilizing and enhancing the unique cleansing action which had been observed previously with the earlier liquid product. The term "ointment-like" has been used since the vehicle incorporating the urea peroxide, while possessing the outward appearance and, in general, the physical properties of an ointment, differs from the usual ointments in that it contains no water. What might be called the continuous phase consists of anhydrous polyhydric alcohols. This has important effects on its pharmacodynamic action, particularly in contact with body fluids such as pus, exudates, blood, and such.

The present preliminary study had as its immediate purpose confirmation of the fact that the ointment retained the ability to give a prolonged release of oxygen in the presence of body fluids which catalyze the breakdown of urea peroxide.

In addition it was felt desirable to attempt to demonstrate by experimental means the cleansing and debriding action reported in preliminary clinical testing. A cleansing agent for the nonintact skin should remove extraneous matter such as dirt and cloth and/or should help free the lesion of pus, exudate, blood, necrotic tissue, and other debris.

In the case of the test ointment, the evolution of oxygen following the urea peroxide breakdown has the mechanical effect of mixing or churning the necrotized material, pus, or exudate so that the material at the bottom of the wound or lesion is brought rapidly to the top. This is designated as wound detergency. A simple experiment has been devised to demonstrate the effectiveness of this churning action.

Procedure for demonstrating detergency. Standard and chemically clean Wasserman tubes were used for this experiment. They were set up in a rack that would hold four test tubes of the same size and diameter. The first tube contained 1 ml of citrated blood and 0.1 g of animal charcoal. To this was added 0.2 g of the ointment under investigation. The second tube was set up in an identical manner with 1 ml of blood and 0.1 g of charcoal, and instead of the ointment 0.2 g of a placebo was used which contained the ointment base but no urea peroxide. The third tube consisted of approximately 1 ml of purulent exudate taken from an infected lesion of a rabbit's abdomen. To this was added 0.1 g of charcoal and

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*T-3 Ointment: Material for this study was furnished by International Pharmaceutical Corp., Philadelphia, Pa.

0.2 g of the ointment. A control tube was used consisting of only blood plus charcoal. All tubes were shaken on a Borner shaker prior to the addition of the ointment so that there was assurance of a completely homogeneous mixture of the blood or purulent exudate and the animal charcoal. After shaking for 2 min the ointments were then added, shaken for another 2 min and allowed to settle and stratify. The results are indicated in Table 1. The noteworthy result of this was the complete stratification of the blood. The charcoal was brought to the surface or even up into the foaming portion of the mixture. After 3 min the entire area of blood or purulent exudate had been freed of more than 90% of the charcoal that was added. The freed charcoal was found in the upper portions of the tube where the foam had become colored.

Table 1. Detergent Action of Experimental Ointment T-3

Tube	Contents	Results	
		Foam layer	Blood layer
I	1 ml citrated blood 0.1 g charcoal 0.2 g T-3 ointment	Black, 4+	Clear
II	1 ml purulent exudate 0.1 g charcoal 0.2 g T-3 ointment	Black, 4+	Clear
III	1 ml citrated blood 0.1 g charcoal 0.2 g ointment base (placebo)	Absent	Black
IV (Control)	1 ml citrated blood 0.1 g charcoal	Absent	Black

Comparison of the antibacterial effect of ointments. In order to observe the comparative effects of various standard ointments with that of the test ointment, an experiment using a modified Bondi and Spaulding technique [8] was conducted. Standard porcelain cylinders 8 mm in diameter were filled with the ointment and placed in the center of a previously seeded petri dish. The concentration of bacteria used for seeding was $5 \cdot 10^8$/ml. The choice of media depended on the growth requirements of the organisms. For most of the pathogens trypticase soy bean agar was used, but where necessary 2% human blood was added as an enriching substance. This was used for the growth of Streptococcus alpha and beta hemolyticus. Five trials were made for each ointment and organism and the results are shown in Table 2. From each inhibitory zone, small squares were cut and subcultured into nutrient broth or brain-heart infusion broth. All cultures were negative for growth.

The zones of inhibition produced by the reaction between the microorganisms and the ointments under investigation should be considered the results of bacteriostatic rather than bactericidal action. The term "antibacterial" is more appropriate and covers the reaction. Since our interest lies primarily with the reaction of a specific chemotherapeutic agent against resistant strains of staphylococci, another experiment was conducted. Six strains of S. aureus, which had been isolated as pure cultures from patients suffering from pyogenic lesions,

Table 2. Comparative Study of Various Ointments on a Series of Pathogenic Microorganisms

Organism	Average size of zone of inhibition for five trials, mm					
	Penicillin, 5000 units/g	Adhesilin, 10,000 units/g	Sulfur (USP)	Sulfadiazine, 5%	Ammoniated mercury, 5%	T-3
Staphylococcus aureus	40	44	0	0	0	50
Staphylococcus albus	24	43	0	0	10	60
Escherichia coli	0	21	0	0	0	36
Streptococcus alpha hemolyticus	32	45	0	0	0	30
Streptococcus beta hemolyticus	32	32	22	0	0	32
Neisseria catarrhalis	34	20	0	34	0	26
Monilia albicans	55	0	0	0	0	56
Pseudomonas aeruginosa	0	0	0	0	0	40
Corynebacterium diphtheriae	26	26	0	0	0	65
Saccharomyces	0	0	32	0	12	70

were tested against varying concentrations of penicillin ointment containing from 10^3 to 10^6 units/g. This was done to establish that the organisms at the time of use were resistant to the action of penicillin in all concentrations. None of the test plates showed any zone of antibacterial activity.

The above experiment was repeated using the same resistant strains of S. aureus against four ointments: penicillin, ammoniated mercury, sulfadiazine, and T-3 (the test ointment). A control was run at the same time using a placebo of the test-ointment base. The technique used was the same as that of the preceding experiment. Results are given in Table 3.

Table 3. Zones of Inhibition* of Five Antibacterial Ointments Tested Against Resistant Strains of Staphylococcus aureus

Ointment	Strain					
	A	B	C	D	E	F
Sulfadiazine, 5%	22	20	16	36	20	22
Penicillin, 5000 units/g	0	0	0	0	0	0
Ammoniated mercury, 10%	10	0	0	0	0	0
T-3	63	62	60	55	64	60
Placebo ointment base	0	0	0	0	0	0

*Expressed in millimeters.

SUMMARY

A preliminary study has been made of a new stabilized urea peroxide in ointment-like form. Its antibacterial properties against ten species of organisms have been measured. Its effectiveness against resistant strains of Staphylococcus aureus when tested by this method has been noted. Its unusual cleansing action has been demonstrated by laboratory techniques.

The product offers interesting possibilities as a new approach to the cleansing of infected and contaminated lesions of the skin and mucous membranes. Its cleansing and debriding action as well as its antibacterial effectiveness may prove to be factors of the greatest importance in its use. Its effectiveness against resistant strains of microorganisms deserves further investigation.

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DISCUSSION

DR. GOSSMAN: Question concerning commercial availability of this agent.

DR. COBE: You can buy it in carload lots, if you want to. It comes from Becco, in Buffalo, and it's a relatively pure, relatively stable compound. The only disadvantage is that, as you well know, hydrogen peroxide is now one of the major things for jet transportation and there are times when it's likely to be in short supply.

ANTIBIOTIC PROPERTIES OF SYNTHETIC LOWER ALKYL AND HALOALKYL PENICILLINS

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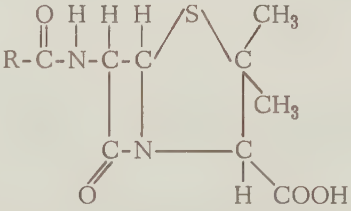
The availability of pure 6-aminopenicillanic acid (6-APA) [1,2] has made possible the synthesis and examination of the antibiotic properties of a variety of penicillins. Of particular interest are those penicillins which cannot be prepared by the classical fermentation techniques or readily isolated from a fermentation broth. In the present work a series of lower alkyl and haloalkyl penicillins was prepared to study the effect of structure on the antibiotic action against representative Gram-positive and Gram-negative microorganisms. The penicillins were tested for in vitro and in vivo activity and those of particular interest were studied for dog and human blood-serum levels.

According to the accepted convention of nomenclature a penicillin is named by the substituent, R, attached to the penicillin nucleus with the general formula as follows:



Thus, a penicillin prepared synthetically by condensation of an organic acid with 6-APA is named according to the radical of the organic acid without the carboxyl group. Hence, the acetyl derivative of 6-APA is called methyl penicillin and likewise the α-chloropropionyl derivative of 6-APA is called α-chloroethyl penicillin.

MATERIALS AND METHODS

The penicillins described in this study were prepared by reaction of 6-APA with the appropriate acid chloride in a basic environment. The crude reaction products were purified, in some cases to a crystalline potassium salt, in other cases to an amorphous freeze-dried product. The purity of the penicillin was determined by the hydroxylamine colorimetric-assay procedure [3], and the antimicrobial properties were reported on the basis of 100% purity.

The in vitro minimum inhibitory concentrations (MIC) were determined using the method described by English et al. [4] by the commonly accepted twofold serial-dilution technique. The in vivo chemotherapeutic efficacy or median protective dose (CD_{50}) in mice was determined as described by English et al. [5]. The microorganisms employed were those used routinely by this laboratory from the Pfizer collection.

The dog and human blood-serum levels were determined by the standard penicillin plate assay. The values for dog blood-serum levels represent averages from five dogs after oral administration of 10 mg/kg body weight. With the human serum levels the values represent the average levels of 15 subjects given 250 mg oral doses on the basis of the free acid of the penicillin.

RESULTS AND DISCUSSION

The in vitro antibiotic properties of the lower alkyl and haloalkyl penicillins are summarized in Table 1. Considered as a group, the penicillins were quite active against the Gram-positive microorganisms tested. The MIC values for the penicillins were in the range of 0.06-0.78 μg/ml against Staphylococcus aureus and 0.02-0.19 μg/ml against Streptococcus pyogenes. Variation of the length of the side-chain or the introduction of various halogen atoms on the side-chain did not produce a graded response on the MIC values. There was, however, a tendency

Table 1. The In Vitro Minimum Inhibitory Concentrations* of Lower Alkyl and Haloalkyl Penicillins

Penicillin	Purity, %	Staphylococcus aureus	Streptococcus pyogenes	Proteus vulgaris	Escherichia coli	Salmonella typhosa
Methyl	50	0.78	0.06	12.5	25	6.25
Chloromethyl	90	<0.19	<0.19	25	50	12.5
Bromomethyl	85	0.38	<0.09	>100	100	12.5
Iodomethyl	65	0.78	0.04	6.25	50	12.5
Ethyl	50	0.19	0.09	12.5	25	6.25
α-Chloroethyl	50	0.09	0.09	100	25	25
α-Bromoethyl	80	0.09	0.09	>100	>100	>100
α-Iodoethyl	55	0.19	0.05	100	100	100
β-Chloroethyl	90	0.19	0.19	50	50	12.5
β-Bromoethyl	60	0.39	<0.19	50	50	6.25
β-Iodoethyl	80	0.39	0.19	50	100	12.5
n-Propyl	60	0.39	0.09	25	50	12.5
α-Chloro-n-propyl	65	0.19	0.02	100	25	25
α-Bromo-n-propyl	70	0.39	0.01	>100	100	100
Isopropyl	75	0.39	0.19	50	25	25
α-Bromoisopropyl	80	0.39	0.01	>100	12.5	>100
α-Iodoisopropyl	55	0.09	0.02	100	50	25
β-Bromoisopropyl	80	0.19	0.02	>100	>100	100
n-Butyl	50	0.19	0.002	25	25	6.25
α-Bromo-n-butyl	95	<0.19	<0.19	>100	100	100
α-Iodo-n-butyl	30	0.09	0.09	100	100	50
α-Bromoisobutyl	75	0.39	<0.19	>100	>100	>100
n-Amyl	85	<0.19	<0.19	50	25	6.25
α-Chloroisoamyl	75	0.09	0.04	>100	>100	>100
α-Bromo-n-amyl	85	<0.19	<0.19	100	100	100
α-Bromoisoamyl	75	0.19	<0.02	100	100	100
α-Bromo-n-hexyl	25	0.06	0.06	100	100	>100
Penicillin G	95	0.03	0.004	50	50	25
Penicillin V	95	0.03	0.02	>100	>100	100

*Expressed in μg/ml.

of the butyl, amylalkyl, and haloalkyl penicillins to give lower MIC values than the methyl, ethyl, or propyl homologs. None of the compounds studied were as active as penicillin G or penicillin V against *S. aureus* or *S. pyogenes*.

Compared to their activity against the Gram-positive bacteria, the structure of the side-chain of the lower alkyl and haloalkyl penicillins had a greater effect on the MIC values of the Gram-negative microorganisms studied. This effect was more pronounced with *Salmonella typhosa* than with *Proteus vulgaris* or *Escherichia coli*, the penicillins being generally less effective against the latter two microorganisms. The alkyl penicillins from methyl to amyl had MIC values against *S. typhosa* of 6.25-12.5 μg/ml except for isopropyl penicillin which had an MIC of 25 μg/ml. With methyl penicillin introduction of a halogen atom on the side-chain had no significant effect on the MIC value. With the α-halosubstituted

Table 2. The In Vivo Properties of Lower Alkyl and Haloalkyl Penicillins

Penicillin	Median protective dose, mg/kg				
	Staphylococcus aureus		Ratio, parenteral/oral	*Salmonella typhosa*	
	Oral	Parenteral		Oral	Parenteral
Methyl	10	2	0.20	>100	90
Chloromethyl	14	11	0.79	300	95
Bromomethyl	>25	20	–	>100	100
Iodomethyl	>25	17	–	–	–
Ethyl	13	3	0.23	>100	90
α-Chloroethyl	4	3	0.75	>100	79
α-Bromoethyl	3	3	1.0	>100	100
α-Iodoethyl	10	8	0.80	>100	100
β-Chloroethyl	4	2	0.50	150	40
β-Bromoethyl	4	2	0.50	70	26
β-Iodoethyl	6	2	0.33	>100	>100
n-Propyl	9	3	0.33	>100	>100
α-Chloro-n-propyl	4.5	2.5	0.55	>100	>100
α-Bromo-n-propyl	11	11	1.0		
Isopropyl	6*	3	–		
α-Bromoisopropyl	4	3	0.75		
α-Iodoisopropyl	18	18	1.0		
β-Bromoisopropyl	8	4	0.50		
n-Butyl	16	6	0.38		
α-Bromo-n-butyl	9	9	1.0		
α-Iodo-n-butyl	13	6	0.46		
α-Bromoisobutyl	5	1	0.20		
n-Amyl	>25	25	–	>400	150
α-Chloroisoamyl	6	6	1.0		
α-Bromo-n-amyl	>25	25	–		
α-Bromoisoamyl	6	6	1.0		
α-Bromo-n-hexyl	14	14	1.0		
Penicillin G	10	1	0.20	>400	238
Penicillin V	1.8	0.8	0.44		

*20% survival.

ethyl penicillins, the chloro analog had an MIC value of 25 μg/ml while the bromo and iodo analogs values were much higher. On the other hand, the β-haloethyl analogs had MIC values in the range of 6.25-12.5 μg/ml. Their in vitro antibiotic activity, thus, is comparable to that of the lower alkyl penicillins. The halopropyl, butyl, and amyl penicillins were less active than their alkyl analog. These data suggest that the more linear alkyl and haloalkyl penicillins are more effective against *S. typhosa* than those with a branched side-chain.

The results of the animal-protection studies for the penicillins against *S. aureus* infections are summarized in Table 2. There does not appear to be any correlation between the CD_{50} and MIC values for the respective penicillins. Changes in the length of the carbon chain or introduction of a halogen atom had little effect on the in vivo activity, except with methyl penicillin, where the introduction of a halogen atom reduced its in vivo activity. The most significant effect was that of enhancement of oral activity relative to parenteral activity by the introduction of a halogen atom. This effect can be noted by a comparison of the ratio of parenteral CD_{50} to oral CD_{50}. With ethyl penicillin the ratio is 0.23 compared to 0.33-1.0 for the haloethyl penicillin analogs. This relative increase of oral activity of the haloalkyl penicillins may be attributed partially to a greater acid stability of the penicillin, but other factors such as the efficiency of absorption and stability against enzymatic degradation may contribute.

The data in Table 2 for animal-protection studies against *S. typhosa* are for penicillins selected from those which had low MIC values. Of this group the β-chloroethyl and β-bromoethyl penicillins were found most effective, giving oral CD_{50} values of 150 and 70 mg/kg and parenteral PD_{50} values of 40 and 26 mg/kg, respectively. On the other hand, the β-iodoethyl, the α-halomethyl, and the alkyl penicillins, which gave comparable MIC values, were markedly less effective. The explanation for the greater in vivo activity of the β-chloroethyl and β-bromoethyl penicillins is not immediately clear but molecular polarity, stability of the halogen side-chain, and stereochemical properties of the side-chain may be among the factors responsible. The relative polarity of the lower alkyl and haloalkyl penicillins can be noted on paper chromatograms. Those penicillins which are active against *S. typhosa* are found to be more polar than penicillin G, whereas those which are much less active against *S. typhosa* are less polar than penicillin G [6]. When compared to penicillin G, many of the lower alkyl and haloalkyl penicillins were found to be more active in *S. typhosa* animal-protection studies.

Results from blood-serum levels of four haloalkyl penicillins given orally to canine and human subjects are summarized in Tables 3 and 4. The penicillins selected were the β-chloroethyl and β-bromoethyl penicillins which were effective against *S. typhosa*, and α-bromoisobutyl and α-bromohexyl penicillins, a highly branched and a linear α-haloalkyl penicillin, respectively.

Table 3. Serum Levels in Dogs After 10 mg/kg Oral Administration

Penicillin	Serum level, μg/ml				
	0.5 hr	1 hr	2 hr	3 hr	6 hr
β-Chloroethyl	0.95	0.71	0.32	0.10	0.04
β-Bromoethyl	1.05	1.01	0.43	0.21	0.01
α-Bromoisobutyl	3.75	2.41	0.46	0.20	0.02
α-Bromohexyl	1.04	0.48	0.20	0.08	0.00
Penicillin V	1.77	1.31	0.33	0.10	0.00

Table 4. Serum Levels in Humans After Single 250 mg Oral Dose

Penicillin	Serum level, µg/ml				
	0.5 hr	1 hr	2 hr	3 hr	6 hr
β-Chloroethyl	0.40	0.48	0.31	0.23	–
β-Bromoethyl	0.21	0.38	0.21	0.01	–
α-Bromoisobutyl	1.25	2.73	0.96	0.48	–
α-Bromohexyl	1.53	1.09	0.28	0.07	–
Penicillin V	1.37	1.83	0.66	0.25	–
Penicillin G	0.29	0.73	0.43	0.25	0.03

Of the penicillins studied the α-bromoisobutyl penicillin gave the highest blood-serum levels both in dogs and in humans. In both cases the levels were higher than those obtained with equivalent doses of penicillin V while the other haloalkyl penicillins studied gave lower blood-serum levels than penicillin V. The β-chloroethyl and β-bromoethyl human serum levels were less than that obtained with penicillin G. This study was too limited, however, to indicate any effect of structure on blood-serum levels for this class of penicillins.

SUMMARY

A series of lower alkyl and haloalkyl penicillins was synthesized to study the effect of structure on the antimicrobial action against selected Gram-positive and Gram-negative bacteria. As a class of penicillins they were found to be effective in vitro and in animal protection studies against Staphylococcus aureus. The β-chloroethyl and β-bromoethyl penicillins were found to be particularly active both in vitro and in vivo against Salmonella typhosa, but less active against Escherichia coli and Proteus vulgaris. Dog and human blood-serum levels were studied for selected haloalkyl penicillins with α-bromoisobutyl penicillin giving levels higher than that obtained with penicillin V.

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DISCUSSION

DR. DONOVICK: Is there any correlation between the structure of the compound and the susceptibility to staphylococcal and B. cereus penicillinases?

DR. SETO: The penicillinases were not studied, but these penicillins are not active against resistant strains of Staphylococcus aureus.

ANTIBIOTIC PRODUCTION BY VARIOUS SPECIES AND VARIETIES OF EMERICELLOPSIS AND CEPHALOSPORIUM

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INTRODUCTION

Recently, considerable attention has been focused on a group of antibiotics called cephalosporins. In 1948 Abraham [1] reported that a fungus, Cephalosporium acremonium, produced an antibiotic which inhibited the growth of a number of bacteria. Later, Burton and Abraham [2] reported that six antibiotics were contained in culture filtrates of Brotzu's Cephalosporium. They also reported that five of the antibiotics were soluble in butyl acetate and that all possessed the basic steroid ring in their structure. These antibiotics were designated as cephalosporin P_{1-5}. The antibiotic remaining in the culture fluid was isolated by Newton and Abraham [3] and was shown to be a penicillin with a D-α-aminoadipic acid side-chain. This antibiotic was called cephalosporin N.

An interesting antibiotic, cephalosporin C, was isolated from a crude preparation of cephalosporin N by Newton et al. [4, 5] and was shown to differ from the latter in several physicochemical properties. Cephalosporin C possesses an unusual modified penicillin nucleus which is resistant to the action of penicillinase and has marked acid stability. Crawford and Abraham [6] reported that the antibiotic also acts synergistically with benzylpenicillin against penicillinase-producing Staphylococcus aureus. For these reasons cephalosporin C is particularly interesting to workers in the antibiotics field.

In 1954 an antibiotic called synnematin was discovered by Gottshall et al. [7] of the Michigan Department of Health Laboratories. The fungus which synthesized the material was identified by Roberts [8] as Cephalosporium salmosynnematum Roberts. Synnematin was resolved into two components, A and B, by Olson et al. [9]. The B fraction was shown by Abraham et al. [10] to be identical with cephalosporin N. Olson and Jennings [11] reported the successful treatment of Salmonella infections in chickens and mice, and Benavides et al. [12] demonstrated the potential of synnematin in the treatment of typhoid fever.

The synnematin-producing organism was obtained by the Department of Botany at the University of Wisconsin, and soon the ascigerous stage of the fungus was discovered by Grosklags and Swift [13]. The mold was placed in the family Eurotiaceae of the Ascomycetes and classified as Emericellopsis salmosynnemata Grosklags and Swift. The proper classification of this organism facilitated collection of molds capable of elaborating antibiotics of the cephalosporin group. This has been reported by Grosklags and Swift [13], Kavanagh et al. [14], Nara and Johnson [15], and Elander [16].

A broad program concerned with natural and induced variability in cephalosporin-producing fungi was initiated at the University of Wisconsin in 1955. The initial studies were confined to C. salmosynnematum MDH48B3UV27, an improved synnematin-producing strain. The subsequent testing of strains derived from this organism gave disappointing results and it appeared that obtaining a markedly superior strain would be a rare event.

In the meantime, another fungus, Emericellopsis terricola var. glabra van Beyma was shown to be a more potent cephalosporin producer. It was decided to use this organism as the parental form in a study of variability in antibiotic-producing fungi, thus extending studies along the same lines employed for Penicillium chrysogenum. The latter has been reviewed by Backus and Stauffer [17].

This paper will report the results of a study of strain variability in regard to cephalosporin production by E. terricola var. glabra and of a study of the capacity of related molds to synthesize an antibiotic capable of inhibiting the growth of certain penicillinase-producing S. aureus isolates.

METHODS AND MATERIALS

The initial approach to the screening of populations grown from ultraviolet-irradiated (2750 A) spores of E. terricola var. glabra followed the pattern established by Backus and Stauffer [17].

Hundreds of isolates, i.e., colonies arising from ultraviolet-irradiated conidia, were processed according to the procedure employed for P. chrysogenum, but eventually this pattern of operation was modified in E. terricola var. glabra for the following reasons: (a) The shake-flask fermentation and assay testing of total survivor populations was so time consuming that only small numbers of isolates could be screened. (b) Although mutants having reduced capacity to synthesize antibiotic appeared in substantial numbers in survivor populations, it eventually became evident that mutation resulting in enhanced capacity to produce antibiotic would be rare. This implied that it would be necessary to screen much larger populations of isolates. (c) The ability to detect anything but a very large change in antibiotic potential by a single shake-flask fermentation test was much less than in the case of the penicillin fermentation.

To accommodate larger populations of isolates a plate screening test was adopted. The procedure for this test was as follows: A diluted suspension of ultraviolet-irradiated spores (ca. 20% survival) was plated onto honey-peptone medium [17]. Hyphal tips were transferred from the margins of 250-500 well-isolated colonies to plates containing 25 ml of Peoria No. 1 agar, two transplants per plate. After 5 days incubation at 32 C, the agar surface of each plate was painted with a 24-hr broth suspension of S. aureus, and the plates were then incubated for 12-18 hr at 37 C. The potency index (diameter of the inhibition zone divided by the colony diameter) was determined for each isolate, and isolates having higher-than-normal index values were retained for further screening in shake-flask fermentations. The normal index value was the average of a number of simultaneously-determined potency indices of colonies originating from untreated conidia.

The fermentation medium was that described by Nara and Johnson [15]. This medium was dispensed in 100-ml quantities into 500-ml Erlenmeyer flasks, plugged, and sterilized in an autoclave for 15 min at 121 C. Upon cooling, each flask was inoculated with a spore-mycelial suspension of the isolate to be tested. The flasks were then placed on a Gump rotary shaker (270 rpm) and incubated at 30 C for 6-8 days. When the fermentation was completed, 2.0 ml of the liquor was aseptically removed from the flask, placed in a small tube, and centrifuged at 5000 rpm. The supernatant fluid was then poured into a test tube and a 0.2 ml sample was diluted with 1% phosphate buffer (pH 6.2) and assayed using the cup-plate method with S. aureus as the test organism. Different dilutions of cephalosporin N of known purity served as standards.

The following procedure was employed for irradiation and the evaluation of strains derived from untreated and ultraviolet-irradiated conidia. Sterile water, 10-15 ml, was poured over the surface of a well-sporulated culture. The suspension was then filtered through Whatman No. 2 filter paper. Spore counts were made with the aid of a Levy haemocytometer and the suspension diluted as required to give finally a concentration of 125-150 spores/mm^3. A 10-ml aliquot of the suspension was transferred to the irradiation vessel. A 0.2-ml sample was also taken immediately and diluted to 50 ml for use later in estimating the percentage of viable spores and as a check of the counting procedure. After the suspension was placed in the vessel and stirred for a short time, and before exposure to ultraviolet radiation, a control sample was removed. All samples were 0.2 ml in volume and were diluted immediately with 100 ml of sterile water. Additional samples were removed at scheduled time intervals during the irradiation.

After irradiation was completed, 1.0-ml aliquots of the untreated control and of the treated samples were pipetted onto the surface of leveled plates containing 20 ml of honey-peptone medium. The plates were then rotated so as to distribute the suspension uniformly over the surface of the plate. Usually, 10 plates were used for each sample. After 3-4 days incubation small colonies were visible. The colonies arising from each sample were counted and a survival curve was plotted.

Colonies arising from a sample of the irradiated suspension in which about 20% of the spores survived the radiation treatment were first screened using the plate screening test. Each isolate was transferred as a small group of hyphal tips from the margin of a colony growing on plates upon which the irradiated spore sample had been plated. No selection was involved at this stage; a group of hyphal tips was cut from each colony. Two such transfers were made to a plate of Peoria No. 1 agar, and this process was continued until 250-500 colonies were transferred. At least 250 transfers were made, to obtain what was considered to be a representative colony population. Usually 20-30 colonies were picked from plates seeded with untreated conidia. These untreated colonies served as controls and on plate tests were used to calculate the normal potency index for the particular strain.

Isolates which had greater potency than the average found for several colonies arising from untreated spores were retained and retested. If the value of the isolate's potency index was confirmed, it was transferred to slants in preparation for further testing in shake flasks. At this stage, an original population was reduced to a small number. Of those colonies selected, some were discarded because of poor growth or a decision to include only those isolates having potency indices falling within a certain maximum range. The relatively few isolates which consistently exhibited higher-than-normal indices in one or more retests would be tested for their capacity to synthesize antibiotic in shake-flask fermentations.

The following Emericellopsis and Cephalosporium entities were examined for inhibitory activity against both penicillin sensitive and penicillin resistant *S. aureus*: *E. terricola* var. *glabra* van Beyma; *E. salmosynnemata* Grosklags and Swift; Emericellopsis sp. n. (WSF No. 47); *Emericellopsis humicola* (Cain) Gilman; *Emericellopsis minima* Stolk; *Emericellopsis mirabilis* (Malan) Stolk; *E. "mirabilis"* (Routien isolate); *E. terricola* var. *terricola* van Beyma; *Emericellopsis* sp. n. (Warcup T15); *C. acremonium* Corda (Brotzu strain).

Hyphal masses of the various isolates were transferred to plates of Peoria No. 1 agar and, after five days incubation, were painted with both penicillin-sensitive and penicillin-resistant Staphylococcus. The isolates were also tested

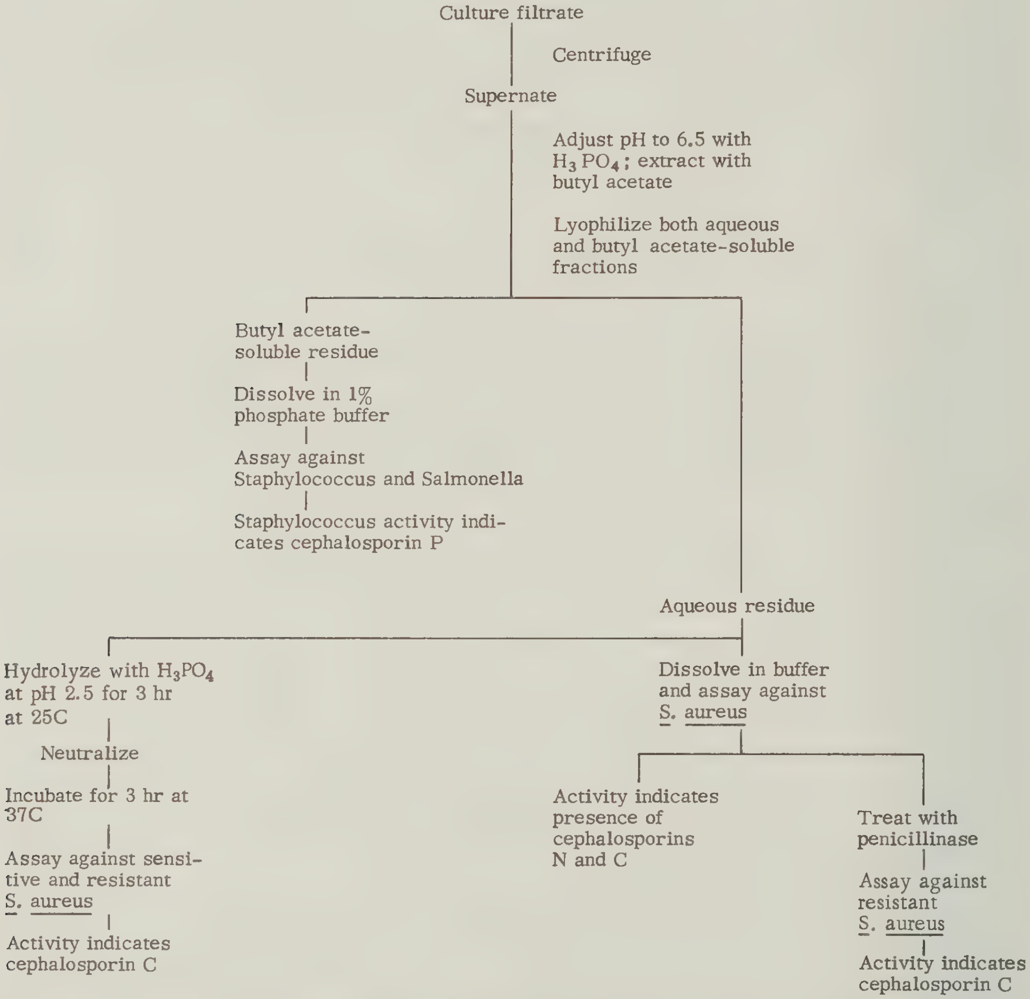

Fig. 1. Scheme for the Detection of Cephalosporin Antibiotics

Culture filtrate
Centrifuge
Supernate
Adjust pH to 6.5 with H_3PO_4; extract with butyl acetate
Lyophilize both aqueous and butyl acetate-soluble fractions
Butyl acetate-soluble residue
Dissolve in 1% phosphate buffer
Assay against Staphylococcus and Salmonella
Staphylococcus activity indicates cephalosporin P
Aqueous residue
Hydrolyze with H_3PO_4 at pH 2.5 for 3 hr at 25C
Neutralize
Incubate for 3 hr at 37C
Assay against sensitive and resistant S. aureus
Activity indicates cephalosporin C
Dissolve in buffer and assay against S. aureus
Activity indicates presence of cephalosporins N and C
Treat with penicillinase
Assay against resistant S. aureus
Activity indicates cephalosporin C

in shake-flask fermentations and assayed according to the procedure in Fig. 1. Later these procedures were modified to incorporate a Vibrio assay method. The latter has been employed at the Antibiotic Research Station, Medical Research Council, Clevedon, England. It is essentially the same as the schedule outlined in Fig. 1. However, the English scheme does not differentiate cephalosporins N and C on the basis of the instability of the latter and the stability of the former to penicillinase. A significant advantage of the English scheme is the use of *Vibrio cholerae* No. 1077 as a test bacterium. *V. cholerae* is highly sensitive to extremely low levels of cephalosporin C commonly found in fermentations of *C. acremonium*. It is capable of detecting levels of cephalosporin C as low as 0.01 units/ml and is apparently unaffected by cephalosporin P. This was described in detail by Bond [18].

V. cholerae is rather fastidious in its growth requirements and it is important to note that optimal growth on a solid medium is obtained only when the agar content is low. The organism has been cultured successfully on the following medium: concentrated papain broth*, 400 ml; Difco yeast extract, 2.5 g; Microelement solution, 1.0 ml; Difco-agar, 7.5 g; distilled water, 600 ml. The final pH was 7.0.

A thin layer of this medium is poured onto a layer of 2% nutrient agar. The surface of the plate is then flooded with a papain broth culture of V. cholerae, and the excess is removed with a pipette. The plates are then placed open in an incubator for 1 hr at 37 C and the filtrate to be tested is added. Since the agar content of the medium is low the filtrates were added to sterile assay discs, dried, and placed on the agar surface. The inhibition zones formed are surrounded by a darker, thicker zone because of abnormal bacterial growth.

The English scheme depends on the destruction of cephalosporin N, i.e., conversion to its corresponding penicilloic acid by acid. While cephalosporin N is destroyed by acid, cephalosporin C is unusually stable, even at a pH below 2.5. The sample to be tested is acidified to pH 2.5-3.0 with M H_3PO_4. It is then incubated at 37 C to destroy any cephalosporin N-penicilloic acid formed. During this incubation it is essential that the pH does not rise above 7.5 or the cephalosporin C is destroyed. The workers at Clevedon state that an acid-stable substance with anti-Vibrio activity is not necessarily cephalosporin C, and point out that confirmatory tests are necessary.

A confirmatory test employed in this study was the inactivation of cephalosporin N by penicillinase. Since penicillinase is a peptidase with high specificity for the β-lactam ring of the penicillin β-lactam-thiazolidine nucleus, and since cephalosporin N has this nucleus, it is rapidly destroyed in the presence of the enzyme. The filtrates which possessed cephalosporin P activity were extracted previously with butyl acetate before the filtrates were treated with acid or penicillinase to obviate interference with tests for cephalosporins N and C.

In chromatographing the filtrates, Na-cephalosporin C, obtained from Dr. E. P. Abraham, Oxford University, 1 mg/ml, and cephalosporin N, obtained from Dr. B. H. Olson, Michigan Department of Health, 100 μg/ml, served as standards. The filtrates were concentrated to about 10 ml and spotted on Whatman No. 1 or No. 3 paper. Known samples of cephalosporin N and cephalosporin C were also spotted and served as reference points. The chromatograms were chromatographed by the ascending method with 70% n-propanol or 95% methanol and buffered at pH 5.5 with 0.013 M sodium citrate. After 12-18 hr the chromatograms were dried and sprayed with 1.5% ninhydrin solution. Other chromatograms were cut in strips and placed on dishes containing Peoria No. 1 agar seeded with S. aureus. After 12-18 hr incubation at 37 C the strips were examined for antibiotic activity, i.e., zones of inhibited bacterial growth along each strip.

RESULTS AND DISCUSSION

In preliminary attempts to identify superior antibiotic-producing strains of E. terricola var. glabra by the plate test, an ultraviolet-induced variant derived from the parent form consistently exhibited higher than normal potency-index values. This variant, Wis. 57-2-34B, was identical to its parent in morphological and cultural characters. When tested for antibiotic production in shake-flask fermentation, correspondingly higher yields were obtained for the variant as compared to the parental strain (Table 1).

Because of its increased capacity to synthesize antibiotic and good vegetative growth compared with that of its parent and other isolates which had been se-

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*Prepared according to the procedure of Asheshov [19].

Table 1. Antibiotic Production by Parental and Wis. 57-2-34B Strains of *E. terricola* var. *glabra*

Strain	Plate assay, potency-index range	Average shake-flask yields, units/ml	
		6 day	7 day
Parental	2.2-2.5	696	854
Wis. 57-2-34B	3.0-3.2	923	1148

lected, Wis. 57-2-34B was chosen as the first "key" strain of the Wisconsin series of *E. terricola* var. *glabra* strains.

As described above, strain Wis. 57-2-34B was subjected to the mutagenic action of ultraviolet radiation. In all, three separate irradiations of this strain were made and in each a population of colonies arising from a sample of irradiated conidia was screened for antibiotic production by the plate method. All strains exhibiting a potency index of 0.2 or more than that of the parent were retained, and then retested and examined for other desirable characters. Some strains were discarded after the second test because of a greatly reduced rate of growth or failure to give a potency index higher than that of the parent strain. The strains retained after the second and sometimes the third screening test were then tested for their capacity to produce antibiotic in submerged fermentation. Finally, considering yield of antibiotic both in plate and shake-flask assays and rate of growth of the colony, six strains were selected for consideration as the parental strain for further selection. These strains together with their parent, *E. terricola* var. *glabra* Wis. 57-2-34B, are listed in Table 2. The six derivatives of *E. terricola* var. *glabra* Wis. 57-2-34B listed in Table 2 were selected from slightly more than 2200 colony isolates. This indicates that the probability of acquiring a superior strain of this fungus through mutagenic treatment, at least with ultraviolet as the agent, is small unless relatively large numbers of survivors can be processed.

If one compares the potency index to shake-flask fermentation yields (Table 2), a good correlation is evident in most cases. Occasionally, however, a strain may show a very high potency index and later on fail to give a correspondingly high antibiotic yield in shake-flask fermentation tests, e.g., *E. terricola* var. *glabra* Wis. 58-3-127A. This strain grows extremely slowly and in such cases the correlation between plate test and shake-flask test is not found.

Table 2. Selected UV-Derived Strains of *E. terricola* var. *glabra*, Wis. 57-2-34B

Strain	Average potency index	Shake-flask yields,* units/ml
Wis. 57-2-34B (control)	3.2	923-1148
Wis. 58-3-180A	3.4	625-717
Wis. 58-3-127A	6.0	756-975
Wis. 58-4-24A	3.5	704-898
Wis. 58-3-236B	4.1	1206-1465
Wis. 58-1-75A	3.8	1282-1735
Wis. 58-4-159B	3.7	1465-1748

*Range for a minimum of 8 flasks per strain.

Of the strains listed in Table 2, Wis. 58-3-236B, Wis. 58-1-75A, and Wis. 58-4-159B appear to be superior to the parental strain, Wis. 57-2-34B, on the basis of their performance in plate assay and shake-flask tests (Figs. 2, 3, 4). With the adoption of the medium described by Nara and Johnson [15], the yields of antibiotic have not only been increased but are also more consistent when testing E. terricola var. glabra type and Wis. 57-2-34B. With these factors in mind, strain Wis. 58-4-159B was selected as the second "key" strain in the Wisconsin series.

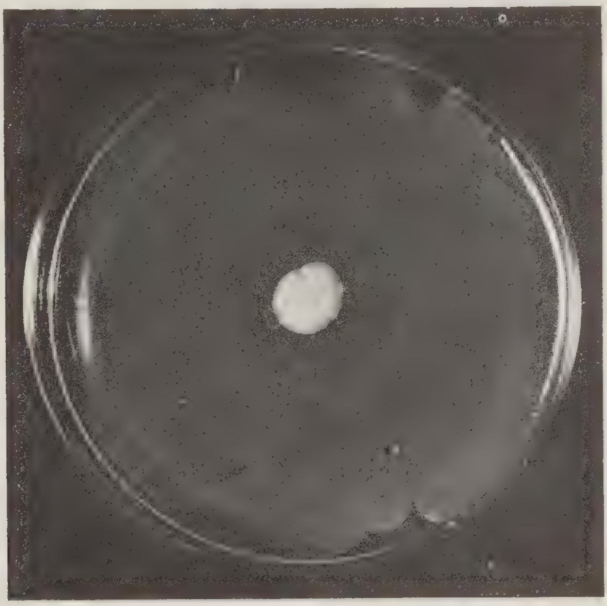


Fig. 2. Antibiotic-producing capacity of E. terricola var. glabra (parent) against Staphylococcus aureus on plate test.

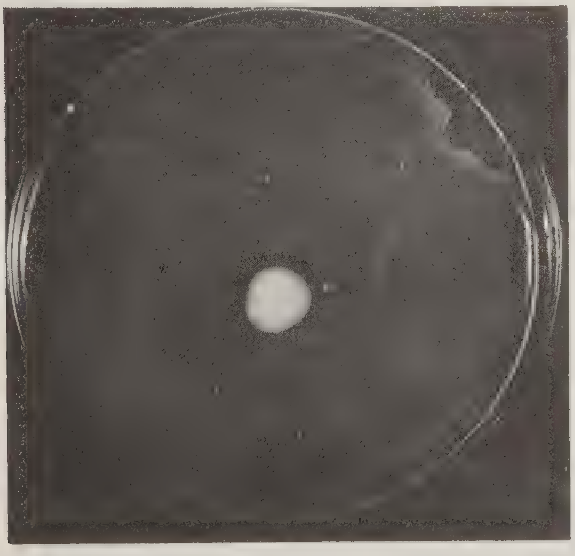


Fig. 3. Antibiotic-producing capacity of E. terricola var. glabra Wis. 57-2-34B against Staphylococcus aureus on plate test.

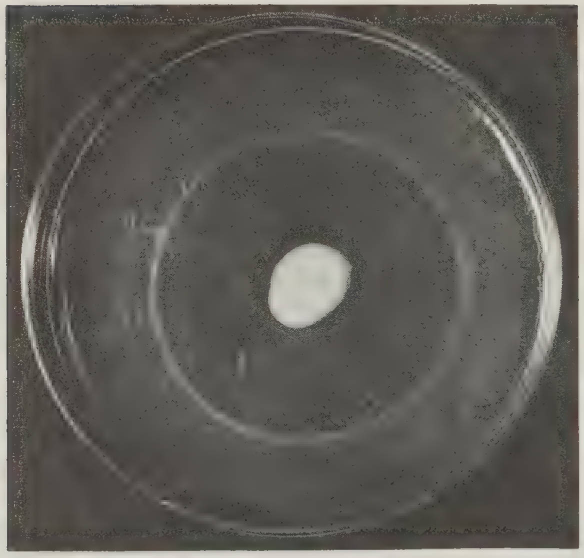


Fig. 4. Antibiotic-producing capacity of E. terricola var. glabra Wis. 58-4-159B against Staphylococcus aureus on plate test.

Following the mutagenic treatment of strain E. terricola var. glabra Wis. 58-4-159B, plate and shake-flask assays of a population of colonies arising from treated conidia yielded several isolates of promise as more potent cephalosporin producers. These are listed along with their potency indices and yields of antibiotic in submerged fermentation tests (Table 3). Wis. 58-5-236B has shown superior yields compared to the other strains of the 58-5 series. This strain has been the most superior isolate encountered and can be considered the third "key" strain in the Wisconsin series. The genealogy of the Wisconsin family of strains of E. terricola var. glabra to date is given in Fig. 5.

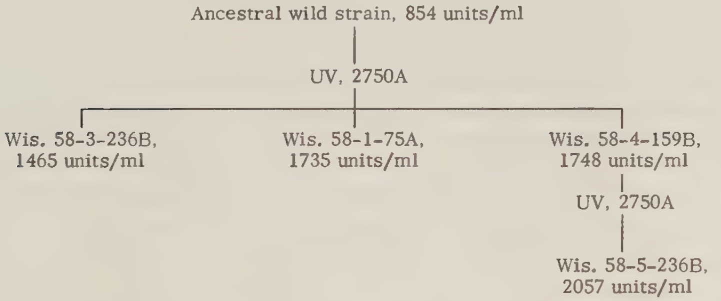
Table 3. Selected UV-Derived Strains of E. terricola var. glabra Wis. 58-4-159B

Strain	Average potency index	Shake-flask yields,* units/ml	
		6 day	7 day
Wis. 58-4-159B (control)	3.70	1486	1992
Wis. 58-5-207A	4.40	1027	1848
Wis. 58-5-32A	3.21	1662	1884
Wis. 58-5-225B	3.50	1673	1820
Wis. 58-5-11A	3.43	1814	1853
Wis. 58-5-236B	3.42	1745	2057

*Maximum yields for two successive tests.

The antibiotic activity of the various species and varieties of Emericellopsis and Cephalosporium toward a sensitive and a resistant strain of S. aureus is summarized in Table 4. These results indicate that a number of Emericellopsis and Cephalosporium entities produce an antibiotic in submerged fermentation capable of inhibiting the growth of resistant S. aureus. It is possible that the activity against penicillin-resistant staphylococci was due to cephalosporin C.

Fig. 5. Wisconsin Family of Strains of Emericellopsis terricola var. glabra.


Ancestral wild strain, 854 units/ml
UV, 2750A
Wis. 58-3-236B, 1465 units/ml
Wis. 58-1-75A, 1735 units/ml
Wis. 58-4-159B, 1748 units/ml
UV, 2750A
Wis. 58-5-236B, 2057 units/ml

Inasmuch as the elaboration of cephalosporin N is widespread in the genus Emericellopsis, the paramount problem was to rule out the possibility that the antibiotic activity against resistant Staphylococcus was not due to high concentrations of cephalosporin N. Abraham [1] has shown that three major cephalosporin antibiotics are elaborated in submerged culture by C. acremonium (Brotzu strain). The three antibiotics, cephalosporins N, P, and C, are different structurally and all three inhibit S. aureus to varying degrees. Cephalosporin P and cephalosporin N inhibit penicillin-resistant Staphylococcus at relatively high concentrations whereas cephalosporin C inhibits these organisms at relatively low concentrations.

A series of experiments was undertaken to test for the presence of cephalosporin P in those filtrates which showed antibiotic activity against resistant Staphylococcus. This antibiotic has been shown to be highly soluble in organic solvents. The filtrates of the various organisms were then extracted with butyl acetate according to the procedure outlined in Fig. 1. These results are summarized in Table 5.

C. acremonium appears to produce an antibiotic which is soluble in butyl acetate. Since cephalosporin P was originally isolated from filtrates of this fungus, there is strong evidence that the activity present in this fraction is cephalosporin P. Kavanagh et al. [14] previously reported on the antibiotics elaborated by four of the Emericellopsis species listed in Table 5. They re-

Table 4. Antibiotic Activity in Fermentation Filtrates of Various Emericellopsis and Cephalosporium Entities

Organism	Oxford cup-plate assay inhibition zone, mm	
	S. aureus, avian strain	S. aureus, phage type 80/81
E. terricola var. glabra	36.0	18.5
E. salmosynnemata	28.5	15.0
E. sp. n. (WSF No. 47)	22.5	13.5
E. humicola	hazy	0.0
E. minima	hazy	0.0
E. mirabilis	12.5	0.0
E. "mirabilis" (Routien isolate)	0.0	0.0
E. terricola var. terricola	0.0	0.0
E. sp. n. (Warcup T15)	37.5	18.0
C. acremonium (Brotzu strain)	27.5	19.0

ported no cephalosporin P production by these organisms under the conditions employed in their laboratory.

Experiments employing paper chromatography were also carried out. The methods employed were those described by Kavanagh et al. [14], who found that cephalosporin P had an R_f value of 0.84-0.94 when n-propanol was used as the solvent. Thin strips of the chromatograms were bio-assayed against *S. aureus*. No cephalosporin P activity was observed for the filtrates of the various *Emericellopsis* entities listed in Table 5.

Table 5. The Production of Cephalosporin P in Shake-Flask Fermentations of Emericellopsis and Cephalosporium

Organism	Antibacterial activity against *S. aureus* of the butyl acetate-soluble fraction
E. terricola var. *glabra*	–
E. salmosynnemata	–
E. sp. n. (WSF No. 47)	–
E. sp. n. (Warcup T15)	–
C. acremonium (Brotzu strain)	+

The various *Emericellopsis* entities were also tested for the presence of cephalosporin N using a chromatographic method. Cephalosporin N standard was also tested and served as a control in the subsequent analyses. The standard yielded a ninhydrin-positive spot corresponding to an R_f value of 0.34-0.44 using the buffered methanol solvent system. Chromatograms of filtrates from the various organisms yielded ninhydrin-positive spots and areas with antibacterial activity similar to those of the cephalosporin N standard, indicating the presence of cephalosporin N and/or cephalosporin C.

Solutions of Na-cephalosporin C, 5 mg/ml, and cephalosporin N, 100 μg/ml, were spotted alone or in combination on Whatman No. 1 paper. When chromatographed with buffered methanol or n-propanol both antibiotics had R_f values of 0.36-0.44, and when chromatographed together, they ran together on the chromatogram. Abraham [1] reported that separation of the two substances is difficult because of the similarity of their molecules.

However, two important alternatives remain for the differentiation of ce-

Table 6. Antibacterial Activity in Crude Shake-Flask Fermentation Filtrates of Various Emericellopsis and Cephalosporium Entities After Hydrolysis With Dilute Acid

Organism	Inhibition zone, mm		
	Sensitive Staphylococcus	Resistant Staphylococcus	*Vibrio cholerae*
E. terricola var. *glabra*	9.5	11.5	12.0
E. terricola var. *glabra* Wis. 58-5-236B	17.5	18.0	18.5
E. sp. n. (WSF No. 47)	26.0	28.0	27.0
E. sp. n. (Warcup T15)	16.5	17.5	18.0
E. salmosynnemata	0	0	0
C. acremonium	19.0	18.0	22.0
Control (standards)			
Cephalosporin N, 500 units/ml	0	0	0
Cephalosporin C, 1 mg/5 ml	12.5	28.0	33.0

Table 7. The Effect of Penicillinase on the Antibiotic Activity in Crude Culture Filtrates of the Emericellopsis and Cephalosporium Entities

Organism	Inhibition zone, mm		
	Sensitive Staphylococcus	Resistant Staphylococcus	Vibrio cholerae
E. terricola var. glabra	9.5	12.5	12.0
E. terricola var. glabra Wis. 58-5-236B	17.0	23.5	18.0
E. sp. n. (WSF No. 47)	15.0	18.5	16.5
E. sp. n. (Warcup T15)	20.0	21.0	21.0
E. salmosynnemata	0	0	0
C. acremonium	26.5	33.0	26.0
Control (standards)			
Cephalosporin N, 500 units/ml	0	0	0
Cephalosporin C, 0.2 mg/ml	15.0	31.5	36.0

phalosporins N and C. Abraham [1] has shown that the two antibiotics can be differentiated by acid hydrolysis and penicillinase treatment.

Ten milliliters of concentrated filtrates of E. terricola var. glabra, Wis. 58-5-236B; E. salmosynnemata Warcup T15, WSF No. 47; and C. acremonium were hydrolyzed at pH 2.75 for 3 hr at 37 C. Culture filtrates of C. acremonium were extracted previously with butyl acetate to remove any cephalosporin P. The filtrates were neutralized with 1 N NaOH and incubated for 1-2 hr at 37 C to destroy any cephalosporin N-penicilloic acid formed. The filtrates were then chromatographed, assayed, or both, against sensitive and resistant S. aureus and V. cholerae. The results of this test are summarized in Table 6.

An acid-stable material appears to be responsible for the activity exhibited by the organisms presented in Table 6. E. salmosynnemata appears to be the only species incapable of inhibiting the three test organisms listed. Since cephalosporin C is also acid-stable it is conceivable that cephalosporin C or a substance with similar activity is responsible for the activity present in the acid-hydrolyzed filtrates of the various Emericellopsis entities. This is further substantiated by the fact that when the acid-hydrolyzed filtrates were chromatographed and bio-assayed slight activity was observed for areas of the chromatograms corresponding to the R_f value of cephalosporin C.

Five-milliliter aliquots of the concentrated filtrates were also treated with penicillinase solution and after 36 hr incubation were assayed against both types of Staphylococcus and against V. cholerae. The results are given in Table 7. The penicillinase tests (Table 7) substantiate the data obtained on acid hydrolysis (Table 6) in that the filtrates of the same Emericellopsis entities exhibiting antibiotic activity after acid hydrolysis also exhibit activity after penicillinase treatment. From the data obtained on acid hydrolysis, penicillinase treatment, and chromatographic separation and bio-assay, there appears to be evidence of production of an acid-stable material with properties similar to cephalosporin C in filtrates of certain members of the Emericellopsis assemblage. It is hoped that more precise information concerning the nature of this material will be forthcoming in the near future.

SUMMARY

In a study of natural and ultraviolet-induced variability in the fungus, Emericellopsis terricola var. glabra, several strains possessed an enhanced capacity to produce cephalosporin antibiotics in both plate and shake-flask fermentation tests. Maximum yields obtainable with *E. terricola* var. *glabra* Wis. 58-5-236B, the most recent member to be included in the Wisconsin series, were nearly two and one-half times those obtained from the original parental stock. The capacity of other species of Emericellopsis and Cephalosporium to synthesize cephalosporin antibiotics are reported. Certain strains were shown to be capable of producing an antibiotic which inhibited the growth of several penicillin-resistant, penicillinase-producing *Staphylococcus aureus* strains. The antibiotic material was acid-stable and resistant to the action of penicillinase. Chromatographic separation and differential bio-assay suggested that the antibiotic was not cephalosporin N or P. In certain respects, the material has several properties in common with cephalosporins C. A schematic analysis for the detection of cephalosporins P, N, and C, and an assay procedure involving *Vibrio cholerae* are also reported.

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LOCAL ECOLOGIC VARIATION IN CLINICAL ISOLATES*

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(Abstract)

Although known to today's scientists, the majority of pathologists, bacteriologists, and clinicians have failed to comprehend the local ecologic variation in clinically isolated microorganisms. The current problem of increasing organismal resistance, which greatly involves the latter groups, requires more localized activity by said groups and the dissemination of such information is paramount in order to hasten a solution. This paper deals extensively with data on microorganismal susceptibility of approximately 15,000 clinical isolates, including both Gram-negative and Gram-positive varieties. Testing by the disc-plate method was carried out with periodic twofold tube-dilution checks. Also presented is specific information as to source of material, type of lesion, and other pertinent clinical data. Graphic illustrations of monthly and seasonal variations are presented to show the practicing physician should constantly utilize bacterial culture and sensitivity tests as a guide for effective antimicrobial therapy. In addition to monthly and seasonal variation, there is a demonstration of significant local (county to county and hospital to hospital within the community) variation. Findings to date definitely discredit the practice of depending upon past experience and/or clinical judgment as the sole criteria for selection of antibacterial therapy.

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*Paper not available at time of publication.

ANTIBIOTIC BACTERICIDAL STUDIES

Bactericidal and Bacteriostatic Tests With Various Antibiotics

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INTRODUCTION

Although the significance of bactericidal activity of an antimicrobial agent in relation to in vivo effectiveness is not known, this property does represent an important biological characteristic of the agent. The determination of bactericidal activity is of considerable scientific importance and it seems desirable, therefore, to employ a procedure for the determination of bactericidal activity which gives reproducible and accurate results. In a previous report from our laboratories [1] the effect of methods on the apparent bactericidal concentration was pointed out. This study indicated that methods utilizing an "all or none" end point, such as the retransfer technique, did not provide meaningful data. The desirability of a procedure based on quantitation of cell death was emphasized.

There is evidence that the rate of death of microbial cells due to bactericidal agents follows the law of mass action [2], therefore, a graph representing concentration of viable cells plotted against time would be an asymptotic curve. In determining thermal sensitivity of microorganisms it is customary to obtain quantitative data on the rate of cell death. Similarly, studies on the death of microbial cells due to chemicals such as ethylene oxide have employed procedures based on the rate of cell death (Grundy et al. [3]). There seems to be no valid reason for considering the death of microbial cells due to antibiotics as fundamentally different from that due to other agents. It would appear desirable, therefore, in antibiotic bactericidal studies to employ a procedure based on the rate of cell death rather than on the presence or absence of viable cells after some period of exposure.

This report presents the results of further studies in our laboratories on the bactericidal activity of various antibiotics and several species of bacteria. The procedure described previously, Grundy et al. [3], and a simplified method were employed. It is hoped that these observations will further emphasize the importance of methodology in determinations of bactericidal activity.

METHODS AND MATERIALS

Procedure for bactericidal and bacteriostatic tests. For the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of an antibiotic, a variation of the twofold tube-dilution test was used, wherein the tubes are continuously agitated during incubation. The agitated system is a method developed by Grundy et al. [1] and offers certain advantages over the usual stationary-tube test. The MBC of the antibiotic ristocetin, for instance, was influenced greatly by the technique employed and it was found that only the shake-tube method gave reproducible results. Screw-cap tubes, 20 × 150 mm, containing 10 ml of brain-heart infusion broth were incubated in a horizontal

Table 1. Bactericidal Action of Bacitracin

Time, hr	Viable cells/ml								MIC	MBC
	Bacitracin concentration, units/ml									
	0	1.56	3.1	6.25	12.5	25	50	100		
	S. aureus J391 inoculum, 188,000 cells/ml									
3	$10.2 \cdot 10^6$	125,000	25,000	13,200	8,250	5,650	1,795	1,850		
6	$1500 \cdot 10^6$	205,000	24,000	4,600	2,690	525	80	50		
24	$160 \cdot 10^6$	$1808 \cdot 10^6$	$275 \cdot 10^6$	$3.8 \cdot 10^6$	125	<5	<5	<5	12.5	12.5
48	$335 \cdot 10^6$	$345 \cdot 10^6$	$280 \cdot 10^6$	$785 \cdot 10^6$	760,000	Sterile	Sterile	Sterile	12.5	25
	S. aureus 698 inoculum, 103,000 cells/ml									
3	$6.2 \cdot 10^6$	43,500	11,500	9,500	3,400	2,150	1,500	1,500		
6	$1125 \cdot 10^6$	149,000	8,050	4,400	500	300	150	150		
24	$790 \cdot 10^6$	$2165 \cdot 10^6$	$105.5 \cdot 10^6$	$1.5 \cdot 10^6$	<5	<5	<5	<5	12.5	12.5
48	$480 \cdot 10^6$	$625 \cdot 10^6$	$1645 \cdot 10^6$	$2785 \cdot 10^6$	Sterile	Sterile	Sterile	Sterile	12.5	12.5
	S. epidermidis 3519 inoculum, 191,300 cells/ml									
3	$1.2 \cdot 10^6$	162,500	88,500	46,000	31,000	14,050	7,200	3,250		
6	$50.5 \cdot 10^6$	82,500	13,850	3,850	2,300	2,350	1,450	1,750		
24	$1040 \cdot 10^6$	375,000	665,000	19,000	45	<5	<5	<5	1.56	12.5
48	$36 \cdot 10^6$	$385 \cdot 10^6$	$315 \cdot 10^6$	13,850	55	Sterile	Sterile	Sterile	6.25	12.5

Table 2. Bactericidal Action of Kanamycin Sulfate

Time, hr	Viable cells/ml							MIC	MBC
	Kanamycin sulfate concentration, µg/ml								
	0	6.25	12.5	25	50	100	200		
S. aureus J391 inoculum, 102,600 cells/ml									
3	$1.7 \cdot 10^6$	415	150	100	< 5	< 5	< 5		
6	$245 \cdot 10^6$	30	20	< 5	< 5	< 5	< 5		
24	$2200 \cdot 10^6$	$690 \cdot 10^6$	$375 \cdot 10^6$	$13.3 \cdot 10^6$	$2.2 \cdot 10^6$	< 5	< 5	25	100
48	$1000 \cdot 10^6$	$1660 \cdot 10^6$	$1250 \cdot 10^6$	$1100 \cdot 10^6$	$395 \cdot 10^6$	Sterile	Sterile	100	100
S. aureus 698 inoculum, 223,300 cells/ml									
3	$1.9 \cdot 10^6$	$1.3 \cdot 10^6$	962,000	627,000	229,000	2,350	60		
6	$38.2 \cdot 10^6$	$10.1 \cdot 10^6$	$7.5 \cdot 10^6$	$4.7 \cdot 10^6$	315,000	5	< 5		
24	$270 \cdot 10^6$	$230 \cdot 10^6$	$490 \cdot 10^6$	$820 \cdot 10^6$	$27.7 \cdot 10^6$	6,200	< 5	100	200
48	$2200 \cdot 10^6$	$260 \cdot 10^6$	$180 \cdot 10^6$	$400 \cdot 10^6$	$720 \cdot 10^6$	$22.5 \cdot 10^6$	Sterile	200	200
S. epidermidis 3519 inoculum, 110,300 cells/ml									
3	$1.4 \cdot 10^6$	4,550	136	< 5	< 5	< 5	< 5		
6	$46.6 \cdot 10^6$	95	< 5	< 5	< 5	< 5	< 5		
24	$1060 \cdot 10^6$	$11.3 \cdot 10^6$	95,000	40	< 5	< 5	< 5	12.5	25
48	$168 \cdot 10^6$	$290 \cdot 10^6$	$27 \cdot 10^6$	2,750	Sterile	Sterile	Sterile	25	50

Table 2 cont.

Time, hr	Viable cells/ml							MIC	MBC
	Kanamycin sulfate concentration, μg/ml								
	0	6.25	12.5	25	50	100	200		
E. coli 729 inoculum, 160,600 cells/ml									
3	$53 \cdot 10^6$	35	< 5	< 5	< 5	< 5	< 5		
6	$2200 \cdot 10^6$	425	< 5	< 5	< 5	< 5	< 5		
24	$2700 \cdot 10^6$	$685 \cdot 10^6$	< 5	< 5	< 5	< 5	< 5	12.5	12.5
48	$1950 \cdot 10^6$	$51.5 \cdot 10^6$	Sterile	Sterile	Sterile	Sterile	Sterile	12.5	12.5
S. typhosa 6539 inoculum, 99,900 cells/ml									
3	349,000	< 5	< 5	< 5	< 5	< 5	< 5		
6	$8.9 \cdot 10^6$	290	< 5	< 5	< 5	< 5	< 5		
24	$1780 \cdot 10^6$	$1.2 \cdot 10^6$	12,550	155	< 5	< 5	< 5	12.5	50
48	$1030 \cdot 10^6$	$1510 \cdot 10^6$	$161 \cdot 10^6$	$7.9 \cdot 10^6$	Sterile	Sterile	Sterile	50	50
P. vulgaris A-JJ inoculum, 84,000 cells/ml									
3	$1.2 \cdot 10^6$	50	50	< 5	< 5	< 5	< 5		
6	$76 \cdot 10^6$	325	< 5	< 5	< 5	< 5	< 5		
24	$325 \cdot 10^6$	$340 \cdot 10^6$	$1800 \cdot 10^6$	$1600 \cdot 10^6$	< 5	< 5	< 5	50	50
48	$700 \cdot 10^6$	$20 \cdot 10^6$	$1000 \cdot 10^6$	$950 \cdot 10^6$	Sterile	Sterile	Sterile	50	50

Table 3. Bactericidal Action of Neomycin Sulfate

Time, hr	Viable cells/ml							MIC	MBC
	Neomycin sulfate concentration, units/ml								
	0	1.56	3.1	6.25	12.5	25	50		
	S. aureus J391 inoculum, 137,500 cells/ml								
3	$3.7 \cdot 10^6$	540	165	50	< 5	< 5	<5		
6	$1290 \cdot 10^6$	290	85	35	20	< 5	< 5		
24	—	—	—	—	$41 \cdot 10^6$	< 5	< 5	25	25
48	$645 \cdot 10^6$	—	—	—	$415 \cdot 10^6$	Sterile	Sterile	25	25
	S. aureus 698 inoculum, 106,000 cells/ml								
3	540,000	371,000	232,000	187,000	54,500	6,150	4,550		
6	$14.3 \cdot 10^6$	>500,000	>500,000	>500,000	18,000	705	120		
24	$580 \cdot 10^6$	$835 \cdot 10^6$	$800 \cdot 10^6$	$500 \cdot 10^6$	$275 \cdot 10^6$	2,150	< 5	25	50
48	$1450 \cdot 10^6$	—	—	—	$320 \cdot 10^6$	$106.5 \cdot 10^6$	Sterile	50	50
	S. epidermidis 3519 inoculum, 210,000 cells/ml								
3	$2 \cdot 10^6$	1,410	260	60	< 5	< 5	< 5		
6	$50.2 \cdot 10^6$	205	15	< 5	< 5	< 5	< 5		
24	$150 \cdot 10^6$	$2.1 \cdot 10^6$	315	< 5	< 5	< 5	< 5	1.56	6.25
48	$102.5 \cdot 10^6$	$590 \cdot 10^6$	460,000	Sterile	Sterile	Sterile	Sterile	3.1	6.25

Table 3 cont.

Time, hr	Viable cells/ml							MIC	MBC
	Neomycin sulfate concentration, units/ml								
	0	1.56	3.1	6.25	12.5	25	50		
	E. coli 729 inoculum, 156,000 cells/ml								
3	$11 \cdot 10^6$	100,000	865	10	5	< 5	< 5		
6	$1780 \cdot 10^6$	$1780 \cdot 10^6$	3,280	310	10	< 5	< 5		
24	$320 \cdot 10^6$	–	$325 \cdot 10^6$	$128 \cdot 10^6$	385,000	< 5	< 5	12.5	25
48	$330 \cdot 10^6$	–	$215 \cdot 10^6$	$15 \cdot 10^6$	$1790 \cdot 10^6$	Sterile	Sterile	25	25
	S. typhosa 6539 inoculum, 88,500 cells/ml								
3	325,000	330	190	< 5	< 5	< 5	< 5		
6	$11.4 \cdot 10^6$	840	215	< 5	< 5	< 5	< 5		
24	$1310 \cdot 10^6$	$205 \cdot 10^6$	12,500	$185 \cdot 10^6$	40	< 5	< 5	3.1	12.5
48	$365 \cdot 10^6$	$1030 \cdot 10^6$	$485 \cdot 10^6$	$170 \cdot 10^6$	$270 \cdot 10^6$	Sterile	Sterile	25	25
	P. vulgaris A-JJ inoculum, 192,000 cells/ml								
3	595,000	106,000	460	<5	< 5	< 5	< 5		
6	$385 \cdot 10^6$	340,000	3,500	140	< 5	< 5	< 5		
24	$535 \cdot 10^6$	$185 \cdot 10^6$	$310 \cdot 10^6$	$185 \cdot 10^6$	30,000	30,000	< 5	50	50
48	$1100 \cdot 10^6$	$330 \cdot 10^6$	$280 \cdot 10^6$	$170 \cdot 10^6$	$26.5 \cdot 10^6$	$165 \cdot 10^6$	Sterile	50	50

position on a reciprocal shaker. The shaker operated at 76 cycles/min with a stroke of 3 in. The test organism was transferred from a refrigerated slant through two 24-hr brain-heart infusion-broth cultures. A 1-ml portion of a 1 : 500 to 1 : 1000 dilution of the second 24-hr broth was added to each tube. Studies in our laboratory showed that this procedure results in approximately 100,000 cells/ml in the inoculated tubes. 0.5 ml of appropriate concentrations of the test antibiotic in water was added to each tube. Viable cell counts were determined after incubation at 37 C, at 3, 6, 24, and 48 hr. To minimize the reduction in volume of the tubes only 0.5-ml samples were removed at each time interval. Appropriate tenfold dilutions were made in deionized water and 1-ml aliquots were plated in duplicate. The plates were poured with 20 ml of B-1 agar in order to minimize the bacteriostatic effect of the antibiotic. B-1 medium, in g/liter, consists of: tryptone, 3; yeast extract, 1; beef extract, 3; glucose, 1; and agar, 15. Sterility tests were made at 48 hr by subculturing 0.1-ml aliquots of the tubes showing no turbidity to duplicate flasks containing 100 ml of fluid thioglycollate medium. The plates used for the determination of viable cell counts and the sterility tubes were incubated for 72 hr at 37 C; longer incubation did not change the results significantly. When penicillin was tested, penicillinase sufficient to inactivate the largest amount of antibiotic used was added to each agar plate. The 48-hr-old shaken tubes showing no visible growth were reincubated after the addition of penicillinase to the tubes and used for a sterility test.

Test organisms. The following organisms were employed: a) Staphylococcus epidermidis Romansky 3519, isolated from acute bacterial endocarditis, penicillin-resistant; b) S. aureus 698, isolated from a wound infection, resistant to penicillin; c) S. aureus Wise J39I [4], resistant to penicillin; d) S. aureus MI-45, isolated from a wound infection; e) S. aureus MI-67, isolated from an infected wound; f) Escherichia coli AC-729, isolated from human source; g) Salmonella typhosa ATCC 6539, resistant to streptomycin; and h) Proteus vulgaris Abbott JJ. These cultures were mainted on tryptose-phosphate agar slants and transferred at least monthly.

Antibiotics used. Stock solutions of the antibiotics were made immediately prior to use and were not used if more than 4 hr old. The antibiotics tested were bacitracin, chloramphenicol, erythromycin, kanamycin, neomycin, novobiocin, penicillin G, streptomycin, and tetracycline.

RESULTS AND DISCUSSION

The nine antibiotics studied are representative of those most commonly used clinically. The MIC and MBC of some of the broad-spectrum antibiotics (kanamycin, neomycin, tetracycline) were determined against Gram-positive and Gram-negative bacteria. This was done in order to determine if the shake method is applicable to both groups of organisms. For a working definition, bactericidal concentration was defined as the concentration of antibiotic killing more than 99.9% of the cells within 48 hr as determined by plate count. In actual practice (Tables 1-5) the tube containing the MBC of a bactericidal antibiotic was usually sterile. To establish the concentration of an antibiotic which is bactericidal, the greater-than-99.9% kill is a more realistic figure than is the requirement of sterility. When plotting the death of cells (exposed to an antibiotic) against time, the curve may well be asymptotic. It would therefore be improper to characterize an antibiotic as bactericidal or bacteriostatic on the basis of a sterility end point. In order for an antibiotic to be considered bactericidal, the difference between the MIC and MBC must be twofold or less. In most cases, this difference was zero;

Table 4. Bactericidal Action of Penicillin Potassium G

Time, hr	Viable cells/ml									MIC	MBC
	Penicillin potassium G concentration, units/ml										
	0	0.03	0.07	0.15	0.31	0.62	1.25	10	100		
	S. aureus 67 inoculum, 99,000 cells/ml										
3	$2.6 \cdot 10^6$	84,500	20,000	17,500	16,500	18,950	18,900	15,900	14,450		
6	$386 \cdot 10^6$	1,450	1,770	2,050	3,600	3,450	3,100	1,850	1,685		
24	$11.5 \cdot 10^6$	285,000	15	15	<5	15	25	5	10	0.03	0.07
48	$400 \cdot 10^6$	$14.5 \cdot 10^6$	1,540	5	<5*	<5*	<5*	<5*	Sterile	0.07	0.15
	S. aureus 45 inoculum, 135,000 cells/ml										
3	$9.9 \cdot 10^6$	325,000	30,000	16,600	12,550	13,800	12,600	7,700	9,400		
6	$1025 \cdot 10^6$	700,000	3,400	2,250	2,900	4,150	5,550	3,900	4,450		
24	$131 \cdot 10^6$	$65.5 \cdot 10^6$	$137 \cdot 10^6$	1,700	3,750	95	80	75	100	0.15	0.62
48	$810 \cdot 10^6$	$31.5 \cdot 10^6$	$112.5 \cdot 10^6$	$206 \cdot 10^6$	5	5	5	<5*	15*	0.31	0.31

*Not sterile

Table 5. Bactericidal Action of Streptomycin Sulfate

Time, hr	Viable cells/ml							MIC	MBC
	Streptomycin sulfate concentration, units/ml								
	0	6.25	12.5	25	50	100	200		
S. *aureus* J391 inoculum, 99,000 cells/ml									
3	$3.8 \cdot 10^6$	1,460	110	< 5	< 5	< 5	< 5		
6	$595 \cdot 10^6$	845	90	< 5	< 5	< 5	< 5		
24	$585 \cdot 10^6$	$775 \cdot 10^6$	$785 \cdot 10^6$	$51.5 \cdot 10^6$	27,000	< 5	< 5	50	100
48	$262.5 \cdot 10^6$	$140 \cdot 10^6$	$600 \cdot 10^6$	$1640 \cdot 10^6$	$10.5 \cdot 10^6$	Sterile	Sterile	100	100
S. *aureus* 698 inoculum, 136,000 cells/ml									
3	77,000	66,500	29,000	5,450	405	300	200		
6	580,000	358,000	76,500	8,100	150	25	< 5		
24	$125.5 \cdot 10^6$	$460 \cdot 10^6$	$415 \cdot 10^6$	$415 \cdot 10^6$	$24.5 \cdot 10^6$	$5.2 \cdot 10^6$	< 5	200	200
48	$260 \cdot 10^6$	$650 \cdot 10^6$	$595 \cdot 10^6$	$430 \cdot 10^6$	$150 \cdot 10^6$	$169 \cdot 10^6$	Sterile	200	200
S. *epidermidis* 3519 inoculum, 173,000 cells/ml									
3	$1.5 \cdot 10^6$	7,100	4,950	2,100	650	500	< 5		
6	$34.5 \cdot 10^6$	6,050	2,250	500	300	< 5	< 5		
24	$665 \cdot 10^6$	89,500	40	< 5	< 5	< 5	< 5	6.25	12.5
48	$228 \cdot 10^6$	$420 \cdot 10^6$	193,000	Sterile	Sterile	Sterile	Sterile	12.5	25

Table 5 cont.

Time, hr	Viable cells/ml							MIC	MBC
	Streptomycin sulfate concentration, units/ml								
	0	6.25	12.5	25	50	100	200		
	E. coli 729 inoculum, 161,000 cells/ml								
3	$3.5 \cdot 10^6$	–	< 5	< 5	< 5	< 5	< 5		
6	$2400 \cdot 10^6$	200,000	30	< 5	< 5	< 5	< 5		
24	$680 \cdot 10^6$	$420 \cdot 10^6$	48,500	< 5	< 5	< 5	< 5	12.5	25
48	$335 \cdot 10^6$	$525 \cdot 10^6$	$1030 \cdot 10^6$	Sterile	Sterile	Sterile	Sterile	25	25
	S. typhosa 6539 inoculum, 39,600 cells/ml								
3	510,000	2,900	770	475	105	10	< 5		
6	$44.5 \cdot 10^6$	10,900	2,390	865	125	20	< 5		
24	$770 \cdot 10^6$	$600 \cdot 10^6$	$855 \cdot 10^6$	$285 \cdot 10^6$	$1.5 \cdot 10^6$	4,050	< 5	100	200
48	$13 \cdot 10^6$	–	–	$245 \cdot 10^6$	$101 \cdot 10^6$	$6.8 \cdot 10^6$	< 5* Not sterile	200	200
	P. vulgaris A-JJ inoculum, 70,300 cells/ml								
3	$1.0 \cdot 10^6$	18,500	550	< 5	< 5	< 5	< 5		
6	$2000 \cdot 10^6$	250,000	245	30	< 5	< 5	< 5		
24	$174.5 \cdot 10^6$	$375 \cdot 10^6$	$900 \cdot 10^6$	$640 \cdot 10^6$	< 5	< 5	< 5	50	50
48	$310 \cdot 10^6$	$162.5 \cdot 10^6$	$105 \cdot 10^6$	$1650 \cdot 10^6$	< 5 Not sterile	< 5 Sterile	< 5 Sterile	50	50

***Flask with 400 units/ml and *S. typhosa* was sterile.**

Table 6. Bactericidal Action of Chloramphenicol

Time, hr	Viable cells/ml								MIC	MBC
	Chloramphenicol concentration, μg/ml									
	0	3.1	6.25	12.5	25	50	100	200		
S. aureus J-391 inoculum, 154,000 cells/ml										
3	$2.4 \cdot 10^6$	270,000	215,000	186,500	123,500	112,000	104,500	72,500		
6	$1270 \cdot 10^6$	335,000	191,000	116,000	71,000	44,000	20,000	20,000		
24	$95 \cdot 10^6$	$730 \cdot 10^6$	36,500	5,450	2,950	1,605	705	570	6.25	>200
48	$575 \cdot 10^6$	$67 \cdot 10^6$	335,000	9,850	1,135	200	90	85	6.25	100
S. aureus 698 inoculum, 103,600 cells/ml										
3	665,000	182,000	158,000	111,500	110,000	104,500	99,500	97,500		
6	$16.4 \cdot 10^6$	373,000	205,000	124,000	124,500	90,500	67,500	63,500		
24	$420 \cdot 10^6$	$102.5 \cdot 10^6$	585,000	89,000	28,500	16,050	8,650	5,650	6.25	>200
48	$380 \cdot 10^6$	$225 \cdot 10^6$	$5.6 \cdot 10^6$	20,750	1,835	1,220	890	715	12.5	>200

Table 6 cont.

Time, hr	Viable cells/ml								MIC	MBC
	Chloramphenicol concentration, μg/ml									
	0	3.1	6.25	12.5	25	50	100	200		
	S. epidermidis 3519 inoculum, 178,600 cells/ml									
3	960,000	263,000	227,500	202,000	218,500	73,000	51,000	34,000		
6	$42 \cdot 10^6$	475,000	218,500	155,000	85,500	17,650	8,000	5,450		
24	$540 \cdot 10^6$	$56.5 \cdot 10^6$	70,000	15,000	3,050	595	325	180	6.25	>200
48	$200 \cdot 10^6$	$46 \cdot 10^6$	760,000	6,000	325	75	30	20	6.25	50
	E. coli 729 inoculum, 145,000 cells/ml									
3	$7.9 \cdot 10^6$	60,500	67,000	60,500	59,000	54,000	58,500	60,500		
6	$3250 \cdot 10^6$	26,000	25,500	37,500	44,000	32,000	25,000	25,000		
24	$2350 \cdot 10^6$	$390 \cdot 10^6$	14,100	9,000	5,000	1,405	730	695	6.25	>200
48	$2200 \cdot 10^6$	$154.5 \cdot 10^6$	18,200	2,950	1,175	250	145	125	6.25	200

Table 7. Bactericidal Action of Erythromycin Base

Time, hr	Viable cells/ml											MIC	MBC
	Erythromycin concentration, μg/ml												
	0	0.19	0.39	0.78	1.56	3.1	6.25	12.5	25	50	100		
				S. aureus J-391 inoculum, 154,000 cells/ml									
3	$6.1 \cdot 10^6$	206,000	232,000	133,000	114,000	87,500	81,500	70,000	68,000	52,000	54,500		
6	$710 \cdot 10^6$	325,000	294,500	78,000	50,000	25,500	23,000	20,000	20,000	16,500	22,000		
24	$795 \cdot 10^6$	$650 \cdot 10^6$	$1.5 \cdot 10^6$	$2.4 \cdot 10^6$	406,000	33,000	850	1,500	1,000	2,000	2,000	1.56	> 100
48	$440 \cdot 10^6$	$69.5 \cdot 10^6$	$68 \cdot 10^6$	$22.5 \cdot 10^6$	705,000	104,000	1,050	1,250	100	50	100	1.56	25
				S. aureus 698 inoculum, 47,500 cells/ml									
3	430,000	131,500	94,500	98,500	81,000	78,000	85,000	84,000	89,500	87,500	64,500		
6	$5.3 \cdot 10^6$	154,500	115,000	75,000	75,500	60,500	61,500	61,000	62,500	64,000	64,500		
24	$960 \cdot 10^6$	360,000	47,000	25,500	16,000	12,350	7,850	8,250	6,300	6,750	5,850	0.19	> 100
48	$1050 \cdot 10^6$	$2300 \cdot 10^6$	113,000	1,600	1,400	1,700	1,150	650	400	700	Sterile	0.39	100
				S. epidermidis 3519 inoculum, 159,500 cells/ml									
3	580,000	310,000	233,500	156,500	148,500	120,000	98,500	79,000	55,000	75,500	54,000		
6	$6.3 \cdot 10^6$	265,000	199,500	165,500	92,500	47,500	25,000	18,000	12,500	9,500	10,000		
24	$1470 \cdot 10^6$	$2100 \cdot 10^6$	$1.2 \cdot 10^6$	106,000	71,000	3,000	800	550	180	35	< 5	0.39	50
48	$65 \cdot 10^6$	$115 \cdot 10^6$	$35 \cdot 10^6$	700,000	186,000	740	75	20	30	5	< 5 Not sterile	0.78	6.25

Table 8. Bactericidal Action of Novobiocin

Time, hr	Viable cells/ml									MIC	MBC
	Novobiocin concentration, μg/ml										
	0	0.39	0.78	1.56	3.1	6.25	12.5	25	50		
	S. aureus J-391 inoculum, 121,000 cells/ml										
3	$3.5 \cdot 10^6$	161,500	145,000	129,500	99,000	100,000	97,500	77,500	78,500		
6	$1110 \cdot 10^6$	44,000	55,500	44,500	32,500	22,800	21,500	14,700	15,300		
24	$295 \cdot 10^6$	550,000	1,580	136,500	4,250	1,405	265	110	150	0.39	>50
48	$345 \cdot 10^6$	$1.7 \cdot 10^6$	223,500	$1.9 \cdot 10^6$	25	55	3,900	<5 Not sterile	<5 Not sterile	0.78	25
	S. aureus 698 inoculum, 31,000 cells/ml										
3	118,500	35,500	43,000	35,000	35,500	34,500	28,000	32,500	36,000		
6	110,000	31,000	29,000	22,400	21,000	16,800	15,900	16,900	14,700		
24	$7.8 \cdot 10^6$	$4.5 \cdot 10^6$	$8.6 \cdot 10^6$	75,500	18,150	4,350	475	1,125	230	0.78	>50
48	$176 \cdot 10^6$	$465 \cdot 10^6$	$355 \cdot 10^6$	$86.5 \cdot 10^6$	505,000	191,500	5,450	365	5	3.1	50
	S. epidermidis 3519 inoculum, 159,500 cells/ml										
3	800,000	216,500	216,000	206,500	180,500	174,500	172,500	154,500	128,000		
6	$48.5 \cdot 10^6$	165,000	124,000	78,000	64,500	64,000	36,500	38,500	49,000		
24	$1120 \cdot 10^6$	$2010 \cdot 10^6$	$1520 \cdot 10^6$	$360 \cdot 10^6$	28,000	79,000	52,500	33,000	4,250	3.1	>50
48	$47.5 \cdot 10^6$	$7.8 \cdot 10^6$	$43 \cdot 10^6$	$45 \cdot 10^6$	310,000	500,000	170,000	24,000	22,000	3.1	>50

Table 9. Bactericidal Action of Tetracycline Hydrochloride

Time, hr	Viable cells/ml									MIC	MBC
	Tetracycline hydrochloride concentration, μg/ml										
	0	0.19	0.39	0.78	1.56	3.1	6.25	12.5	25		
	S. aureus J391 inoculum, 149,000 cells/ml										
3	—	222,000	200,000	125,000	102,000	94,500	85,000	50,500	52,000		
6	—	207,500	163,000	77,000	54,500	25,500	21,950	16,650	11,400		
24	—	—	30,000	5,150	3,100	2,230	1,500	995	750	0.39	>25
48	$74 \cdot 10^6$	$910 \cdot 10^6$	46,500	290	305	385	275	135	90	0.39	12.5
	S. aureus 698 inoculum, 73,500 cells/ml										
3	320,000	174,500	153,000	93,000	84,500	72,000	92,000	85,000	80,000		
6	$4.6 \cdot 10^6$	370,000	193,000	107,000	80,500	76,500	56,000	42,000	61,000		
24	$350 \cdot 10^6$	$41 \cdot 10^6$	$3 \cdot 10^6$	35,000	12,900	8,750	4,900	2,890	2,180	0.78	>25
48	$640 \cdot 10^6$	$255 \cdot 10^6$	$174.5 \cdot 10^6$	23,000	525	635	585	505	310	0.78	>25
	S. epidermidis 3519 inoculum, 190,000 cells/ml										
3	865,000	255,000	203,000	235,000	164,000	99,500	88,500	55,000	51,000		
6	$19 \cdot 10^6$	315,000	223,500	200,500	118,000	53,500	23,150	12,500	7,800		
24	$2410 \cdot 10^6$	$31 \cdot 10^6$	285,000	66,000	20,500	3,650	1,450	830	550	0.39	>25
48	$147 \cdot 10^6$	$18.1 \cdot 10^6$	$55.5 \cdot 10^6$	15,650	1,840	470	220	115	70	0.78	>12.5

Table 9 cont.

Time, hr	Viable cells/ml								MIC	MBC
	Tetracycline hydrochloride concentration, μg/ml									
	0	1.56	3.1	6.25	12.5	25	50	100		
	E. coli 729 inoculum, 161,000 cells/ml									
3	$9 \cdot 10^6$	149,000	117,500	124,500	106,500	103,000	32,500	13,350		
6	$2050 \cdot 10^6$	68,500	45,500	78,500	98,500	60,500	12,550	680		
24	$295 \cdot 10^6$	$1.4 \cdot 10^6$	82,500	69,000	27,000	9,050	250	<5	1.56	100
48	$260 \cdot 10^6$	$200 \cdot 10^6$	215,000	56,500	30,000	2,450	305	Sterile	3.1	100
	S. typhosa 6539 inoculum, 36,600 cells/ml									
3	396,000	42,500	31,000	27,500	30,000	17,000	4,150	<5		
6	$4 \cdot 10^6$	13,200	22,000	30,500	29,500	16,200	1,900	<5		
24	$1260 \cdot 10^6$	785	460	1,720	6,700	7,300	890	<5	1.56	100
48	$630 \cdot 10^6$	129,500	85	335	2,100	2,500	495	Sterile	1.56	100
	P. vulgaris A-JJ inoculum, 108,000 cells/ml									
3	$1.9 \cdot 10^6$	57,000	85,000	107,000	81,000	68,500	3,650	2,000		
6	$1080 \cdot 10^6$	865,000	40,000	81,500	62,000	64,500	445	155		
24	$190 \cdot 10^6$	$60 \cdot 10^6$	$40 \cdot 10^6$	$15 \cdot 10^6$	23,500	18,000	<5	<5	12.5	50
48	$285 \cdot 10^6$	$45 \cdot 10^6$	—	—	101,500	4,450	Sterile	Sterile	12.5	50

the main exception was with the S. epidermidis 3519 culture which consistently showed a twofold difference between the MIC and the MBC (Tables 1-4).

Inoculation of approximately 100,000 cells/ml was an arbitrary decision; larger or smaller numbers of cells have been used by other workers [5,6]. It was felt that 100,000 cells/ml is a reasonable approximation of cell numbers in systemic infections and is also large enough so that a resistant mutant may emerge.

For routine testing of the bactericidal property of an antibiotic against a given organism, a simplified shake-tube method can be used. One tenth milliliter samples may be plated from each tube at 24 and 48 hr and only plates showing less than 300 colonies are counted. The 48-hr sterility test plus the plate-count data are sufficient to determine the activity of the antibiotic. This simplified method maintains the advantages of the more detailed shake-tube test but requires only a minimum of time and effort.

Tables 1-5 summarize the results obtained with the bactericidal antibiotics, bacitracin, kanamycin, neomycin, penicillin G, and streptomycin, respectively. These antibiotics meet the requirements of a twofold difference or less between the MIC and MBC and a greater-than-99.9% kill. Kanamycin, neomycin, and streptomycin (Tables 2, 3, and 5) are bactericidal against the Gram-positive and Gram-negative bacteria and indicate that the shake-tube technique is useful with a wide variety of microorganisms. This is in contrast to the results with the stationary tube-dilution test [1] which show extreme variation in the bactericidal activity of an antibiotic when tested against different organisms.

It is interesting to note that P. vulgaris, S. typhosa, E. coli, and S. epidermidis, when tested with neomycin (Table 3), formed atypical, small colonies on agar plates. These colonies were less than 0.5 mm in diameter and developed from tubes containing 25 to 50% of the MIC of neomycin. Some of the colonies were isolated by transfer to agar slants. However, after one to two transfers on antibiotic-free medium the cultures reverted to their normal size. The small colonies exhibited the same sensitivity to neomycin as did the parent cultures and are therefore not resistant variants but bacterial persisters [5,6].

Neomycin at 50% MIC against all six organisms showed a dramatic rate of kill. All organisms showed cell counts of less than 1000 cells/ml at 3 or 6 hr with 50% MIC. However, each culture was able to recover and at 24 or 48 hr showed cell populations sufficient to cause gross turbidity in the tube.

Penicillin, as shown in Table 4, is bactericidal against the two S. aureus strains. However, although death of the cells exceeded 99.9% none of the tubes was sterile. This occurred even when the concentration of penicillin was more than 300 times greater than the MIC and may explain why penicillin has been reported as being bactericidal or bacteriostatic [7]. Again, this emphasizes the importance of methodology and quantitative definitions.

Tables 6 to 9 summarize the results obtained with the bacteriostatic antibiotics, chloramphenicol, erythromycin, novobiocin, and tetracycline, respectively. The MBC obtained with the antibiotics is 8 to greater than 32 times more than the MIC, a significant difference when compared to the bactericidal antibiotics. Tetracycline (Table 9) against S. typhosa and novobiocin against S. aureus J391 (Table 8) showed the zone phenomenon [8,9]. The bacterial cells were more readily killed near the MIC than at higher concentrations.

SUMMARY

The bactericidal activity of nine widely used antibiotics has been reported. The agitated-tube method has been shown to give meaningful and reliable data in

evaluating the bactericidal activity of antibiotics. Furthermore, this method is applicable with a variety of Gram-positive and Gram-negative bacteria. For routine tests, a simplified test has been described which will give the information required with a minimum of time and effort.

Differences between bactericidal and bacteriostatic antibiotics have been determined quantitatively. For an antibiotic to be called bactericidal, a greater-than-99.9% kill and a difference of twofold or less between the MIC and MBC is required. Bacitracin, kanamycin, neomycin, penicillin G, and streptomycin have been classed as bactericidal antibiotics while chloramphenicol, erythromycin, novobiocin, and tetracycline are primarily bacteriostatic in their action.

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DISCUSSION

DR. BONDI: I wonder if I might start the discussion by asking Dr. Otto if he would like to comment about the practical value in a routine hospital laboratory of testing the bactericidal activity of antibiotics against a pathogenic microorganism.

DR. OTTO: I think the implication may be that there is some relationship between the in vitro activity and the in vivo use of an antibiotic. In this particular case, we don't mean to imply that because an antibiotic acts in a certain way in an in vitro system it may act the same way in vivo.

DR. BONDI: Is it not true, Dr. Otto, that an antibiotic which is bactericidal for one of a given group of sensitive microorganisms is uniformly bactericidal to all these strains? Conversely an antibiotic that is bacteriostatic in its activity, is uniformly so to all sensitive strains. Although one might argue in favor of the use of a cidal agent in chemotherapy over a static agent, it is probably not necessary to go through the extra steps that would be needed to test the cidal activity uniformly against all organisms isolated in a hospital laboratory.

DR. SYLVESTER: I don't think anyone has ever demonstrated on a scientific basis that there is any correlation between clinical efficacy and bactericidal and bacteriostatic properties. As a matter of fact this is something that you cannot demonstrate because you can't carry out an in vitro study in vivo. I don't think that whether or not an antibiotic is bactericidal or bacteriostatic is at all important in a hospital laboratory. I don't think this property is significant in terms of the information that the physician needs to choose the antibiotic for the management of his particular patient. I do feel, however, that if anyone is going to study antibiotics or antimicrobial agents from a scientific standpoint, that they need to use a technique which is scientifically sound in order to describe the characteristics of the antibiotics with respect to whether it is bacteriostatic or bactericidal. In our laboratory, our feeling is very strong in that we object to the characterization of an antibiotic as bactericidal on the basis of a retransfer technique from bacteriostatic set up tubes. From a clinical standpoint, I do not feel that there is necessarily any correlation between bactericidal properties and clinical effectiveness. I think it would be too expensive to try to carry out this operation routinely in a hospital laboratory.

DR. ROMANSKY: We discussed some aspects of this in relation to clinical practice this morning. And I do think, however, that there is one point that was made this morning that should be emphasized again this afternoon. These studies which Dr. Otto described do reveal, however, that there is a place for increasing the dosage of antibiotics and that depending on the height or concentration of the antibiotic one would have a decrease in the number of surviving cells. This morning we pointed out that in so many instances, and even in severe infections, the doses of standard antibiotics are kept at the same standard 1- or 2-levels, without increasing that dose either by the intravenous route or other routes. I would modify in one respect Dr.

Sylvester's point about the significance of bactericidal testing from a clinical standpoint. I believe that with patients with sepsis or endocarditis, we must do some form of bactericidal testing, at least to get some indication during therapy as to whether or not the patient's serum containing the antibiotic is doing the job it should, not at a suppressive level, but at a killing level. Now you will not necessarily eliminate all the cells, but one thing we must remember is that the chemotherapeutic agent isn't the only agency functioning and that we have the host as well as the parasite and the drug to consider. It isn't just the drug alone that produces the end result.

DR. SAZ: I would like to ask two questions, one theoretical, one practical. The theoretical question is a very old one. What can be visualized on a mechanistic basis as the difference between bacteriostatic and bactericidal activity? Is it a question of two different mechanisms being inhibited or is it just the same mechanism and a lot more of an agent is needed to achieve killing.

I wonder if the difference between bacteriostatic and bactericidal is not a question of definition. I wonder if you could give the definition in another manner so that we could find closer agreement between the two. Suppose instead of taking 99.9% killing you take 50% or 45% for the definition of bactericidal action and geared your bacteriostatic definition accordingly. Would that make a real difference? The question again is, from a mechanistic point of view, how is an antibiotic working, how do you distinguish between bacteriostatic and bactericidal action? Do you think they are hitting, for example, the same enzymatic system in the organisms or in bactericidal action do you have to hit many more such systems?

DR. OTTO: I can't add anything to that. I think you have covered most of the possibilities that might exist in this case. We thought it was unfair to call an antibiotic bacteriostatic simply because there were a small number of cells surviving after an arbitrary time limit of 48 hr, which we selected and used here. I think other people have used other criteria. I think the point is that we want to get down to the level where we have a small number of cells in relation to the amount of inoculum that we are testing. For that reason, we selected the 99.9% end point. I don't know what would have happened with our results had we selected 50% end points as you suggested.

DR. SYLVESTER: This is a very interesting question and it is this particular question that led us into this work initially. We felt that the techniques and the approaches to the characterizing of antibiotics as bacteriostatic or bactericidal were sloppy. There was no cognizance of the fact that there are chemical reactions involved in the death of a cell which follows the law of mass action. I think that actually the definition of bacteriostatic and bactericidal is purely an arbitrary one. If you look at the kinetics of the death of a cell in the initial part of the curve and plot concentration of viable cells against time, the slope of the curves is fairly uniform for varying concentrations. It is only when you reach the point on the basis of the law of mass action when the reaction is slowing down that there appears to be a difference in the rate between one substance and the other. And it is in this area that we are attempting to define bactericidal and bacteriostatic. Now, I personally feel that this is an arbitrary distinction and we can only define bactericidal in terms of some criteria. In other words we have to define what we are going to use as a basis. We are not holding that our criteria are necessarily the correct ones, but the criteria that we have selected seem to fit the practical observations that have been made with respect to the designation of antibiotics. At the ACS meeting Drs. Humphrey and Nickerson from MIT presented a paper on thermal-death curves. Dr. Humphrey undertook this study as a result of a challenge at an earlier meeting. He had assumed initially that thermal-death-time data were logarithmic, an assumption that is prevalent in textbooks on microbiology. Interestingly enough, using temperatures sinusoidally in relation to thermal-death time, he concluded that the data are nonlogarithmic in nature. This brings the thermal-death time into the same area as the killing of microbial cells by a chemical agent. At this particular moment, I personally am not sure that there is any basic difference in the reactions that are involved or the mechanism of what happens between cells killed by exposures to certain temperatures and those killed by exposure to various chemical agents.

DR. GRIFFITH: Don't we have clinical evidence that bactericidal actions are evident clinically? When we increase the doses of penicillin we are more effective in increasing our killing rate. You might say that this is due to increased penetration into the area by higher doses. In the treatment of bacterial endocarditis, as Dr. Romansky brought out, we use two antibiotics and the clinical effectiveness parallels the ability of the person's serum to kill.

A: I think it is very important to know which antibiotics are bactericidal and which are bacteriostatic in sensitivity testing. This is often overlooked, because if you look at the test of your organisms after 6 hr you might have an indication that you would want to use this bacteriostatic antibiotic, but if you don't look at your test until 12 or 15 hr or longer it may overgrow, and you will overlook this antibiotic.

DETERMINATION OF ANTIMICROBIAL ACTIVITY BY A RADIOISOTOPE METHOD*

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INTRODUCTION

The present report is designed to show the potential of a radioisotope method for (a) rapidly determining antimicrobial activity and (b) studying the interaction between bacteria and an antimicrobial agent. The method is adapted from one used by Levin et al. [1] for the detection of coliform organisms in water.

In principle, the method is essentially the same as any other respirometric technique in which the amount of CO_2 released as a result of metabolism is measured. The method described here consists of seeding a growth medium containing radioactive carbon with bacteria, introducing antimicrobial agents into some of the media, collecting the CO_2 which is evolved, and comparing by means of the tracer the amount of CO_2 to that evolved from antibiotic-free controls. Inhibited cells yield less CO_2 than noninhibited cells.

MATERIALS AND METHODS

Twenty-five-millimeter planchets normally used in standard counting equipment are sterilized and used as culture dishes. Introduced into each culture planchet is 0.8 ml of trypticase-soy broth which contains 0.003% C^{14}-sodium formate (specific activity = 2 mC/mmole, which results in 0.8 μC/planchet). One set of culture planchets is not seeded but serves as a sterile control to determine if any nonmetabolic $C^{14}O_2$ is released from the medium. One set of planchets is seeded with the test organism but is kept free from antibiotics in order to serve as a positive control. Other sets of culture planchets are seeded and have various concentrations of antibiotic added. The final volume of liquid in each planchet is 1.0 ml. The planchets are then incubated at 37 C.

At the end of the desired incubation period, planchets similar to those used for culturing the test organism but containing a paper pad soaked with saturated $Ba(OH)_2$ are inverted over the culture planchets, thereby forming a small collection chamber. The $C^{14}O_2$ diffuses to the pad and is precipitated as $BaCO_3$. The pad containing the labeled $BaCO_3$ is dried directly in the planchet holding it and the amount of radioactivity is determined using standard counting equipment.

Tube-dilution determinations are made concurrently, using the same concentrations of broth, antibiotic, and inoculum.

All of the bacteria thus far investigated have been isolated from patients at Georgetown University Hospital and the numbers shown in the tables are hospital identification numbers.

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*This investigation was supported in part by a Public Health Service research grant, E-3119.

Table 1. Effects of Antibiotics on $C^{14}O_2$ Released by a Staphylococcus (7232) in $3\frac{1}{2}$ hr

Antibiotic	Concentration	Counts/min	Tube dilution, 24 hr
None		1147	
Penicillin	1 unit/ml	1969	Growth
	5 units/ml	1784	Growth
	50 units/ml	190	Growth
Chloramphenicol	1 μg/ml	1332	Growth
	10 μg/ml	69	No growth
	50 μg/ml	59	No growth
Tetracycline	1 μg/ml	2486	Growth
	5 μg/ml	2538	Growth
	50 μg/ml	45	No growth
Erythromycin	1 μg/ml	59	No growth
	5 μg/ml	70	No growth
	50 μg/ml	58	No growth

Concentration of cells, 10^{-2} dilution of an 18- to 20-hr culture. Counts/min have background and sterile controls subtracted and are averages of duplicates.

RESULTS

Table 1 presents the results obtained when cells were incubated for 3 hr and the $C^{14}O_2$ was collected subsequently for 30 min. It can be seen that there is complete correlation between the radioisotope method and the tube-dilution determinations. This particular Staphylococcus was completely resistant to penicillin. It exhibited resistance to 1 and 5 μg of tetracycline and only to 1 μg of chloramphenicol. It appeared to be sensitive to all of the concentrations of erythromycin.

The trend of metabolic activity and $C^{14}O_2$ evolution can be observed by collecting the $C^{14}O_2$ over two different time periods (Table 2). It can be seen that the positive control activity shows a considerable increase over the 2-hr interval. The same kind of reaction was observed in all cases where the corresponding

Table 2. Effects of Antibiotics on $C^{14}O_2$ Released by Escherichia coli (10979) in 2 and 4 hr

Antibiotic	Concentration	Counts/min		Tube dilution, 24 hr
		2 hr	4 hr	
None		1915	15,810	
Penicillin	1 unit/ml	2654	25,406	Growth
	5 units/ml	1379	23,105	Growth
	50 units/ml	4665	9,854	Growth
Tetracycline	1 μg/ml	273	1,588	Growth
	5 μg/ml	348	16	No growth
	50 μg/ml	17	12	No growth

Concentration of cells, 10^{-2} dilution of an 18- to 20-hr culture. Counts/min have background and sterile controls subtracted and are averages of duplicates.

Table 3. Effects of Antibiotics on $C^{14}O_2$ Released by Proteus (10967) over 6 hr

Antibiotic	Concentration	Counts/min						Tube dilution 24 hr
		1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	
None		230	1,500	9,805	27,982	10,839	6,243	
Penicilin	1 unit/ml	200	1,362	8,820	19,766	18,020	3,967	Growth
	5 units/ml	193	1,274	7,552	30,625	14,690	6,403	Growth
	50 units/ml	248	3,441	18,597	28,605	8,545	1,556	Growth
Tetracycline	1 μg/ml	167	401	721	2,613	5,286	17,118	Growth
	5 μg/ml	154	144	311	418	1,781	9,543	Growth
	50 μg/ml	88	37	28	14	11	10	No growth

Concentration of cells, 10^{-2} dilution of 18- to 20-hr culture. Counts/min have background and sterile concontrols subtracted and are averages of duplicates.

tube dilutions became positive, indicating a lack of sensitivity of the test organism. Decreasing amounts of $C^{14}O_2$ were obtained over the 2-hr interval where the corresponding tube-dilution tubes remained negative.

The length of time over which $C^{14}O_2$ was collected was extended to 6 hr with collections made at 1-hr intervals (Table 3). $C^{14}O_2$ collection was maintained for 15 min between each 1-hr interval. The antibiotic-free control reached a peak at 4 hr and the bacteria exposed to the penicillin all followed the same pattern of $C^{14}O_2$ evolution. The corresponding tubes in the tube-dilution determination were positive when read the following day. The effect of the tetracycline on this particular Proteus culture was different from the effect of penicillin. There apparently was some degree of inhibition even at a concentration of 1 μg, as indicated by the lag in $C^{14}O_2$ evolution, although there was a break at 4 hr with a subsequent lack of sensitivity of the culture. The same effect observed

Table 4. Effects of Antibiotics on $C^{14}O_2$ Released by a Staphylococcus (6018) in $4\frac{1}{4}$ hr

Antibiotic	Concentration	Counts/min	Tube dilution, 24 hr
None		1450	
Penicillin	1 unit/ml	1189	Growth
	5 units/ml	1333	Growth
	50 units/ml	113	Growth
Chloramphenicol	1 μg/ml	1222	Growth
	10 μg/ml	25	No growth
	50 μg/ml	23	No growth
Erythromycin	1 μg/ml	27	No growth
	5 μg/ml	32	No growth
	50 μg/ml	28	No growth
Tetracycline	1 μg/ml	39	Growth
	5 μg/ml	31	No growth
	50 μg/ml	26	No growth

Concentration of cells, 10^{-2} dilution of an 18- to 20-hr culture. Counts/min have background and sterile controls subtracted and are averages of duplicates.

at 5 μg concentration was even more pronounced than at 1 μg. However, at 50 μg there was a constant decrease in $C^{14}O_2$ evolved, clearly demonstrating the effect of time as well as drug concentration on the reaction of the culture to the tetracycline. Again this correlated with the results of the tube-dilution determination.

The data shown in Table 4 illustrate the importance of the time of collection of the $C^{14}O_2$ if only a single determination is made. As can be seen, there was complete correlation with the results of the tube-dilution determinations with the exception of the values at 1 μg of tetracycline. On the basis of the 6-hr studies shown in Table 3 one would assume that the amount of $C^{14}O_2$ increased later at the 1 μg concentration. However if one considers the results of any of the antibiotics as a block or unit, sensitivity or lack of it can still be determined.

The problem of cell concentration versus antibiotic concentration and time of determination is present in the radioisotope method just as it is in the conventional methods. This is illustrated by the data in Table 5. It can be seen that the activity detected at 4 hr was not sufficient to determine sensitivity but that at 5 or 6 hr the trends correlated with the tube-dilution determinations. The low control values point to the problem of generally low activity and would prevent a false determination of sensitivity.

Table 5. Effects of Antibiotics on $C^{14}O_2$ Released by Achromobacter (10949) over 6 hr

Antibiotic	Concentration	Counts/min						Tube dilution, 24 hr
		1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	
None		0	2	5	3	89	174	
Penicillin	1 unit/ml	0	5	6	4	18	70	Growth
	5 units/ml	3	2	6	0	41	85	Growth
	50 units/ml	0	2	6	2	49	98	Growth
Tetracycline	1 μg/ml	0	5	30	24	29	80	Growth
	5 μg/ml	4	3	10	0	20	79	Growth
	50 μg/ml	1	5	3	4	4	10	No growth

Cell concentration, 10^{-2} dilution of 18- to 20-hr culture. Counts/min have background and sterile controls subtracted and are averages of duplicates.

DISCUSSION

The data presented are representative of a number of determinations which show that the sensitivity of some bacteria can be determined readily within 4 hr either by using a $C^{14}O_2$ collection at 2 and 4 hr and observing trends or, in some cases, with a single collection. The most reliable determinations are those in which trends are observed and it is this method which is favored by the authors. There are data which show that in some cases 1- and 3-hr determinations are satisfactory although none of these have been included in this report.

By collecting $C^{14}O_2$ early and continuing to collect at regular intervals it is possible to observe the interaction between the culture and the antibiotics. In this way one can see the effect of time and it is possible that with some variations in the method bacteriostatic or bactericidal effects of the antimicrobial agents could be determined within a relatively short period of time.

Although a number of Gram-positive and Gram-negative bacteria have been investigated there is still a need for more to be studied. There is also a need for a greater degree of standardization of size of inoculum so that all bacteria react within 3 or 4 hr.

Investigations are being carried out presently to study the above problems and the direct application of the radioisotope method to body fluids. In this connection some studies have been done on urine specimens and, in the limited number of specimens tested, good results have been achieved in determining sensitivity directly. However, there appears to be a lack of correlation between $C^{14}O_2$ released and the numbers of bacteria present. The investigations are being continued.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Mr. Paul D'Amato and Mrs. Doris Curran for their assistance with this investigation.

SUMMARY

The antimicrobial activity of various antibiotics is determined by using a radioisotope method. The data show that antibiotic sensitivity of bacteria isolated from patients can be determined within 4 hr and are in agreement with results obtained from tube-dilution sensitivity tests done concurrently. The application of the radioisotope method to the direct determination of sensitivity of bacteria in material freshly obtained from patients is being investigated currently.

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DISCUSSION

DR. BONDI: Dr. Heim, do I understand that potentially you could test any organism by this method? Do all organisms have this enzyme system? What is the enzyme? What is the mechanism?

DR. HEIM: We selected formate because of the fact that it is a single-carbon compound. There are many ways that organisms can do things to formate, making it open to the possibility that it would be universally used. Naturally, we haven't tested all organisms. We have done roughly 100 determinations of this sort and we have yet to find one that doesn't work, and this includes Dr. Waksman's favorites, the streptomycetes. They and all the bacteria that we have tried, both Gram-negative and Gram-positive, will do it. The rate of evolution of carbon dioxide will vary even from one staphylococcal strain to another.

DR. BONDI: What about a Gonococcus?

DR. HEIM: We haven't tried it.

DR. CARR: I wonder if I could ask some technical questions here. What kind of counter are you using?

DR. HEIM: Window counter. Nuclear-Chicago model with an M5 changer.

DR. CARR: I understand you incubate your organisms. Is the culture vessel covered while you incubate it?

DR. HEIM: We do this and run duplicates in a petri dish. We have tried covering it with large cover slips and we find that it makes very little difference for our purposes.

DR. CARR: And then you place something containing barium hydroxide on top of this culture planchet?

DR. HEIM: Yes, an inverted planchet which contains only a 25-mm-diameter paper pad soaked with barium hydroxide solution.

Q: I suspect that you are not recommending this for routine use at the present time. The techniques involved would probably go beyond the capabilities of most clinical bacteriology laboratories. But I think that this is certainly a commendable form of enterprise and I wonder if you would comment on the technical problems involved? How difficult is it to carry out one of these procedures on a routine basis? It is apparent too that you can't use this with an organism that is growing anaerobically?

DR. HEIM: The ease or the simplicity of the test possibly is best illustrated if I tell you that we have used this sort of thing for two years now in our medical student's laboratory. We actually have the medical students carry out the reaction.

DR. SAZ: How do you explain the fact that even with low concentrates of antibiotics you are getting much higher counts in the media than you are in the controls?

DR. HEIM: We think this may depend on the size of the inoculum. As I am sure you realize, this is an extremely sensitive method of detection.

Q: Let's assume that the antibiotics are inhibiting the growth of the culture. Therefore one would assume that any acid which is produced would be minimal under such a situation. Now, in the control without antibiotics the organisms are growing and conceivably are producing acid. In such a circumstance might you not lose CO_2 to the atmosphere because of the lower pH in the controls? In the antibiotic-containing planchets you would not be losing CO_2 to the atmosphere as much because the pH is not decreasing. Therefore, you might expect under these circumstances to have a higher count, not that they actually produce more but because they have lost less from the culture medium.

DR. HEIM: Except that you might also have this evolution going on while you are collecting the labeled CO_2.

Q: Except for very fastidious organisms a tube-dilution test or a disc test can give you sensitivity information on many organisms in, let's say, 6 hr. What does this simple sensitivity test do that a conventional disc test does not do?

DR. HEIM: The way it is set up now, nothing. We think it has potential as a laboratory tool to study trends or interactions. It doesn't tell you any more but shortens the period of time in some cases. I don't want to become involved in a definition of the value of bacteriostatic versus bactericidal, but there is the possibility that this can be shown within a few hours by this method.

Q: Don't you think it's important that in four hours you can tell whether or not an antibiotic is effective on the organism?

DR. HEIM: In some cases, yes. In other cases the time that you start an antibiotic isn't lifesaving. If a hospital or medical center had facilities for doing these tests quickly, clinicians who normally do not use sensitivity tests might be willing to do so if they could have results by the end of the day.

ANTISTAPHYLOCOCCAL ACTIVITY OF SERA AFTER ORAL DOSES OF DEMETHYLCHLORTETRACYCLINE, TETRACYCLINE, AND A COMBINATION OF TETRACYCLINE AND NOVOBIOCIN*

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The role of antibiotic combinations in medicine has been a subject of considerable discussion in recent years. At the Fourth Annual Symposium on Antibiotics in 1956 [1] there was noted a "distinct trend toward combined therapy." Support of this "third era of antibiotics," however, has not been unanimous. While many advocate combined antibiotic therapy, some object to the fixed ratio of the antibiotics combined in the single dosage forms. Also, some combinations of particular antibiotics seem to be of questionable value. Finland and his colleagues have compared the action of many antibiotics and their analogues used singly with the action of combinations containing them. One such combination reported recently by Hirsch and Finland [2] was novobiocin and tetracycline. Their data showed lowered novobiocin activity when it was given in combination with tetracycline. There was also a slight suggestion in their data that the tetracycline activity was likewise depressed when given in combination with novobiocin; however, this was not certain since matched doses of tetracycline alone were not administered.

The present study is a comparison of the antistaphylococcal activity of human serum after administration of matched doses of tetracycline, demethylchlortetracycline, and a tetracycline-novobiocin combination.

MATERIALS AND METHODS

The antibiotics used in this study were in the form of capsules coded to keep their identity unknown to the subjects and the authors until the study was completed. The contents of the capsules were as follows: tetracycline phosphate complex, 250 mg (tetracycline hydrochloride equivalent); a mixture of tetracycline phosphate complex, 250 mg; and novobiocin sodium, 125 mg; and demethylchlortetracycline, 150 mg.†

Sixteen healthy young adults, 14 males and 2 females, whose weights varied from 64 to 104 kg (average 80 kg), were the subjects in this study. No subject was receiving any medication other than the test materials at the time of study. Nine individuals, in cross rotation, were given single doses at weekly intervals as follows: 500 mg of tetracycline (2 capsules); 500 mg of tetracycline plus 250 mg of novobiocin (2 capsules); and 300 mg of demethylchlortetracycline (2 capsules). Each dose was given in the morning after breakfast. Blood was obtained immediately before ingestion of the capsules and at 2, 4, 6, and 24 hr later. Three additional individuals were fasting when they received these drugs.

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*This study was supported, in part, by a grant from The Upjohn Company, Kalamazoo, Michigan.

†The trade name of The Upjohn Company for this tetracycline is Panmycin, and for this novobiocin-tetracycline combination, Panalba. The trade name of Lederle Laboratories Division, American Cyanamid Company, for demethylchlortetracycline is Declomycin.

They took breakfast after the 2-hr sample. Otherwise they were comparable to the above group. Another group of four individuals received larger doses. Two were given 750 mg of tetracycline (3 capsules) and two others 750 mg of tetracycline plus 375 mg of novobiocin (3 capsules). The next week the regimen was reversed. This group was also fasting and was comparable to the above groups in other respects.

Serum from each blood sample was separated, coded, and frozen until assays were performed, except for the last group of four individuals. These sera were harvested and assayed on the same day that the last specimens were collected. All sera from the same subject were tested simultaneously.

Four strains of Staphylococcus aureus were used to test the activity of the sera. One, S. aureus 209P (ATCC 6538P), was susceptible to both tetracycline and novobiocin. Of three other strains recently isolated in this laboratory, one each was susceptible to one antibiotic but not the other and one was resistant to both.

Dilutions of the sera were made by adding 0.5 ml of each serum to a tube containing 0.5 ml of trypticase soy broth. In pilot experiments Bacto brain-heart infusion broth had shown much less growth. Therefore, the size of the inoculum was reduced to one drop of a 1/1000 dilution of a 24-hr culture of the test organism delivered from a 30 gauge needle. The results were read and recorded according to the method described by Jones and Finland [3].

An additional check on the sera of the fasting subjects receiving 3 capsules each was made by using the cup-plate method. The 2-, 4-, and 6-hr specimens drawn following ingestion of each of the preparations in the double crossover were diluted in the six cups of one plate after the method given by Grove and Randall [4] for tetracycline assays. The zone sizes around the three cups containing serum specimens of tetracycline were then compared with the zone sizes around the three cups containing the tetracycline-novobiocin serum specimens. S. aureus 209P was used in the seed layer.

RESULTS

Of the four used, the only test organism which was inhibited consistently was S. aureus 209P. The other three were never completely inhibited although occasionally the tetracycline and demethylchlortetracycline sera permitted less

Table 1. Relative Activity of Sera From Normal Subjects Against Staphylococcus aureus 209P After Single Oral Doses of Tetracycline, a Tetracycline-Novobiocin Combination, and Demethylchlortetracycline

Number and type of subject	Dose,* mg	Average levels of activity†					Average peak level ± SD	Average total activity first 6 hr ± SD
		0 hr	2 hr	4 hr	6 hr	24 hr		
9, Nonfasting	500T	0	3.3	3.4	3.3	0	3.6 ± 0.5	16.8 ± 3.4
	500T+250N	0	2.4	3.2	2.5	0.1	3.3 ± 0.8	13.7 ± 3.2
	300D	0	0.2	0.2	0.1	0	0.2 ± 0.3	0.8 ± 1.1
3, Fasting	500T	0	2.8	2.7	1.7	0	3.2 ± 0.2	12.7 ± 1.7
	500T+250N	0	1.8	2.2	2.8	0.2	2.8 ± 0.6	10.8 ± 5.1
	300D	0	2.0	2.0	2.3	0	2.5 ± 0.8	10.3 ± 5.9
4, Fasting	750T	0	3.8	4.1	4.6	0.1	4.6 ± 0.7	20.4 ± 1.0
	750T+375N	0	2.6	3.8	3.4	0.3	4.0 ± 0.8	16.1 ± 2.7

*T = Tetracycline, N = Novobiocin, D = Demethylchlortetracycline

†Expressed as maximum number of twofold dilutions of serum that inhibited S. aureus 209P

growth of the tetracycline-susceptible novobiocin-resistant organism than of the tetracycline-resistant novobiocin-susceptible one. None of the sera had any significant effect on the doubly resistant organism.

No difference in activity was noticed in the sera which were frozen until assayed and those which were not frozen but assayed promptly.

The zones of inhibition in the cup-assay method were larger around the cylinders containing sera following ingestion of tetracycline alone than around those containing sera obtained after ingestion of the combination. The test doses were administered in rotation among the 12 subjects of the triple crossover study in such a way that all six possible orders were carried out in duplicate. There was no indication that the sequence of the doses influenced the results nor could weight be correlated with titers.

The average levels of activity in the sera expressed as the maximum dilution are shown in Table 1 along with the average peak levels and total activity for the first 6 hr after each dose. Total activity is expressed in dilution hours, which are equivalent to the area under the curve of the serum levels. These curves and the individual values in each experiment are shown in Fig. 1.

A comparison of the total activity (dilution hours) in the group of nine non-fasting subjects shows significantly less activity resulting from a dose of 500 mg of tetracycline plus 250 mg of novobiocin than with 500 mg of tetracycline given alone ($P < 0.05$). In only one individual was the activity greater (2.5 dilution

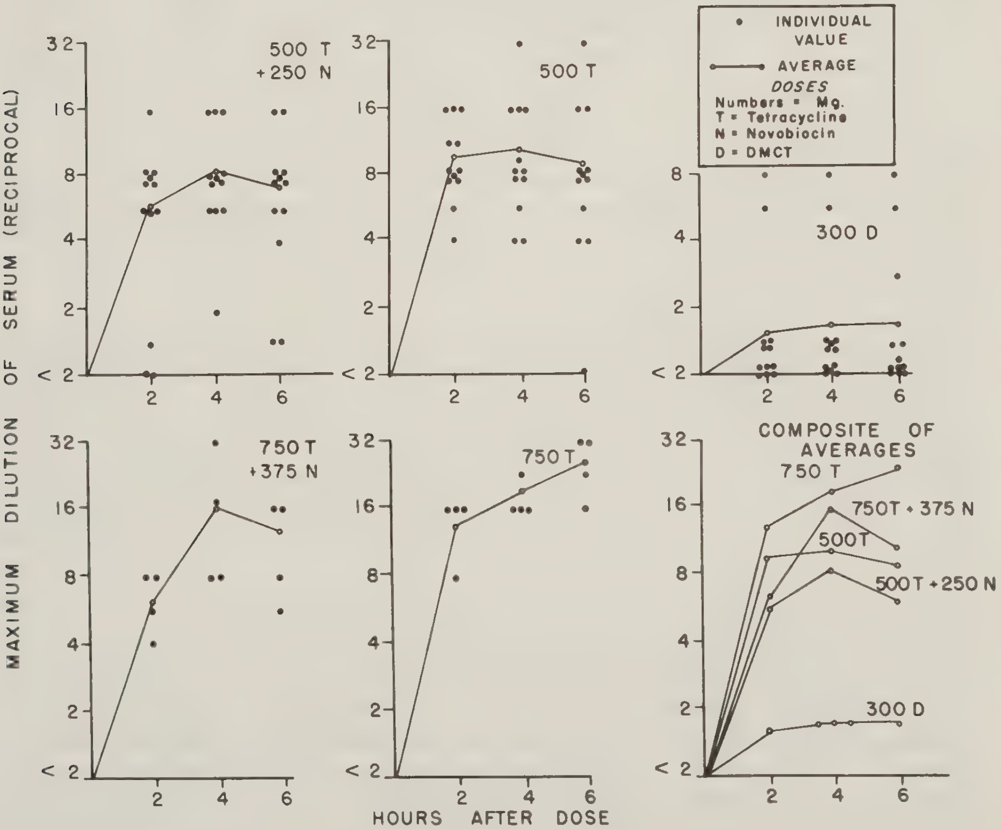

Fig. 1. Inhibition of Staphylococcus aureus 209P by sera after ingestion of tetracycline, demethylchlortetracycline, or a tetracycline-novobiocin combination.

hours) for the combination. The addition of novobiocin appears to have resulted in a suppression of the activity of tetracycline in the other eight persons. Similar reduction of the activity of the tetracycline-novobiocin combination is seen in the group of three fasting subjects and in the group of four other fasting subjects who received higher doses despite the fact that S. aureus 209P is somewhat more sensitive to novobiocin than to tetracycline.

Demethylchlortetracycline appeared to have no appreciable activity in the sera of nonfasting subjects. In fasting subjects 300 mg of demethylchlortetracycline produced activity less than 500 mg of tetracycline alone and roughly equal to that of 500 mg of tetracycline plus 250 mg of novobiocin in combination. No appreciable demethylchlortetracycline activity was detected at 24 hr.

DISCUSSION

The data presented here show, as suggested by the work of Hirsch and Finland [2], a depression of the activity of tetracycline when given in combination with novobiocin, despite a number of in vitro studies reporting synergism between these two antibiotics [5, 6]. The present study was concerned with the tetracycline-novobiocin combinations as compared with tetracycline alone and one of its analogues. The omission of doses of novobiocin alone in the experiment was intentional since such studies by others were known to be in progress. For example, Foltz and Graves [7] determined the serum concentration of tetracycline and novobiocin after these antibiotics were administered alone and in combination. Serum levels were lower following ingestion of the combination than those after either drug was taken alone. This was not true, however, when the combination was administered as separate capsules of the components instead of in one combination capsule.

While synergism was not demonstrated in this study, other reasons for the use of this particular combination have been proposed. Among these are control of mixed infections [8], prevention of emergence of resistant strains which occurs readily when novobiocin is used alone [9], and reduction of the problem of staphylococcal entercolitis, which is the most frequent serious complication when tetracycline is administered alone [10].

In several previous reports demethylchlortetracycline was found to produce higher and more sustained levels of serum antibacterial activity when compared with the older tetracycline analogues on a weight-for-weight basis. In two such reports Finland et al. [11] compared demethylchlortetracycline with tetracycline, chlortetracycline, and oxytetracycline on the basis of serum antibacterial activity after a single dose, and with tetracycline given in repeated doses over a 7-day period. On the basis of these and similar studies the assumption was made that demethylchlortetracycline in the amount of 150 mg was as good or better than 250 mg of tetracycline.

Nevertheless, Perry et al. [12] found that a single dose of 300 mg of demethylchlortetracycline produced less serum antibacterial activity than 500 mg of tetracycline in a group of fasting subjects whose blood levels were tested over a 24-hr period. They concluded that the greater activity of demethylchlortetracycline against the test organism was not adequate to compensate for the smaller amount ingested. In a subsequent study Roberts et al. [14] tested serum antistreptococcal activity following single doses of 300 mg of demethylchlortetracycline and 500 mg of tetracycline. The tetracycline produced significantly greater activity over a 24-hr test period. When 150 mg of demethylchlortetracycline or 250 mg tetracycline was administered q.i.d. for 4 days there was less serum activity for the first day with demethylchlortetracycline, and even after 4 days serum activity with demethylchlortetracycline was less than with tetracycline. There was no significant "extra day" activity with either drug. They demonstrated that demethylchlortetracycline was heavily bound by serum and that the greater activity of demethylchlortetracycline in broth media was completely nullified by the addition of serum. These authors suggest that serum binding may inhibit the in vivo effectiveness of demethylchlortetracycline to a significant extent.

Our results fail to show any advantage for demethylchlortetracycline when it is compared with tetracycline under the conditions of this study. Indeed, a single 300-mg dose of demethylchlortetracycline appears to produce less activity than 500 mg of tetracycline. Recent reports of light sensitivity produced by demethylchlortetracycline further detract from its usefulness [13].

In a group of nonfasting subjects demethylchlortetracycline produced only negligible serum activity. The most reasonable explanation would seem to be the one advanced by Roberts et al. [14] that marked binding of the drug by serum inhibited its activity. Since serum antistaphylococcal activity after ingestion of demethylchlortetracycline was much greater in fasting subjects than in nonfasting, the increased serum binding may be the result of, or enhanced by, the meal.

The cup-plate method does not permit a complete evaluation of the combined action of two antibiotics because of the difference in diffusion rates. Nevertheless, the test was made in this case since no increased activity of the combination was expected. The results with both agar diffusion and broth dilution showed suppression of tetracycline activity by novobiocin.

Some nonfasting subjects were used in this study, in contrast to the customary use of fasting subjects only, since the therapeutic use of these preparations is usually without regard to meals. Also, the techniques used here have differed slightly from those used by Finland, yet the resulting titers and their standard deviations are comparable on a dosage basis.

ACKNOWLEDGMENTS

The cooperation of Dr. Elwood L. Foltz, University of Pennsylvania Hospital, Philadelphia, and Dr. William M. M. Kirby, University of Washington School of Medicine, in the exchange of unpublished data was greatly appreciated. The technical assistance of Marlene A. Baker (research technician) was most valuable. The assistance of Betty Sue Boosveld (laboratory assistant) and John E. Siegmund (Junior Research Trainee sponsored by the Baltimore Biological Laboratories, Inc.) is also gratefully acknowledged.

SUMMARY

Oral administration of 500 mg of tetracycline in a combination capsule with 250 mg of novobiocin resulted in lower serum antistaphylococcal activity than administration of 500 mg of tetracycline alone in nine nonfasting and three fasting subjects. Similarly, when doses of 750 mg of tetracycline and 375 mg of novobiocin in combination and 750 mg of tetracycline alone were given to four other fasting subjects the activity resulting from tetracycline alone was greater. Demethylchlortetracycline (2 capsules of 150 mg each) seemed to have greater activity in fasting than in nonfasting subjects but much less than either of the other two antibiotic preparations.

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DISCUSSION

DR. KIRBY: I would like to compliment Dr. Greer on his nice paper. I would like to comment especially on the aspect about the Declomycin. In contrast with practically all published studies showing tetracycline equivalents, the activity of Declomycin seems much lower than tetracycline in this study employing a tube-dilution test and showing antistaphylococcal activity. This is exactly the result that we have observed with tube-dilution studies against streptococci. We have also separately observed the effect of serum that was described by Dr. Greer. As Dr. Greer said, this same phenomenon is present with tetracycline but much less prominently and to a lesser degree than with the demethylchlortetracycline.

MUTUAL INTERFERENCE OF TETRACYCLINE AND NOVOBIOCIN IN COMBINATION

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INTRODUCTION

Among the theoretical indications for combined antibiotic therapy, mixed infections and the attempt to delay the emergence of resistant organisms have had some medical justification and acceptance. These indications among others motivated pharmaceutical companies to formulate fixed combinations of antibiotics. Such fixed combinations have fallen into general disfavor with leading therapists but they enjoy widespread use by physicians at large.

Early reports [1,2,3] suggested that the combination of tetracycline with novobiocin would enhance the activity of these antibiotics, especially against staphylococci, and fixed combinations were made available in several dosage forms. Although some authors suggested that the combination of these two antibiotics decreased the degree of resistance developed by staphylococci, others could not demonstrate any appreciable delay in the emergence of resistant organisms [4,5].

Because of our interest in pharmacological problems related to antibiotic therapy we undertook a series of studies which revealed a pattern of absorption indicating some mutual interference between novobiocin and tetracycline as evidenced by bio-assay methods. These studies were well underway when Hirsch and Finland [6] using serum dilutions demonstrated significantly less antibacterial activity after the oral administration of a 2 : 1 mixture of tetracycline and novobiocin than from a similar dose of novobiocin alone. Wright, using agar diffusion studies of duplicate serum samples obtained from Hirsch and Finland, also noted reduced novobiocin activity when the two antibiotics were given instead of novobiocin alone [6].

Initially we concerned ourselves with the effects of the parenteral administration of these antibiotics, alone and in combination, and subsequently with corresponding oral forms. Our early results led to the later experiments designed to further explore and explain this interference.

MATERIALS AND METHODS

Healthy adults chosen either from a prison population or from a group of hospital and laboratory workers served as subjects. They received various parenteral and oral forms of novobiocin and tetracycline, alone and in combination. A Latin square crossover design was used, each individual receiving one or both of the agents singly in addition to receiving each of the combinations in a given experiment.

Novobiocin was available as the acid, the sodium salt, or the calcium salt. Tetracycline was available as the base, the hydrochloride, or the phosphate. Several combinations were also available.*

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*Medications were prepared and supplied by The Upjohn Company, some of the medications corresponding to Upjohn products, Panmycin ®, Albamycin ®, and Panalba ®.

Serum concentrations of the antibiotics were estimated from the antibacterial activity measured by a modified cup-plate agar-diffusion method of bio-assay. Results are reported in μg/ml but the reader should remember that what is meant is activity corresponding to such a concentration. Serum for these studies was obtained from venous blood samples taken immediately prior to administration of test doses and at intervals designated in the tables reporting results. The test organism for the novobiocin assay was Staphylococcus albus ATCC 12,228, resistant to tetracycline. The test organism for tetracycline was Staphylococcus aureus U-607, resistant to novobiocin.

In the novobiocin study, serum samples were diluted with equal parts of phosphate buffer (pH 4.5) and the resulting zones of inhibition were compared with a standard curve obtained with novobiocin sodium in equal parts of serum and buffer. In the tetracycline study, serum samples were diluted with two parts of 5% gelatin solution (pH 4.5) and the resulting zones of inhibition were compared with a standard curve obtained with tetracycline hydrochloride diluted with the gelatin solution.

An experiment was performed to compare serum concentrations following intramuscular injections of novobiocin alone and in combination with tetracycline. Five subjects participated in this experiment. The doses are shown in Table 1.

A second experiment compared serum concentrations following intramuscular injections of three combinations of novobiocin and tetracycline, tetracycline alone, novobiocin alone, and serum concentrations following the oral ingestion of novobiocin and tetracycline in combination. Twelve subjects participated in this experiment. The injected combinations contained tetracycline base and novobiocin sodium. Tetracycline injected alone was in the form of the phosphate and novobiocin injected alone was in the form of the free acid. Diluents and doses varied (Table 2).

A third experiment compared serum concentrations obtained with 2:1 and 1:1 mixtures of tetracycline and novobiocin in oral liquid form. No comparison was made with the antibiotics taken alone. Twelve subjects participated in this experiment (Table 3).

Another experiment compared serum concentrations following the oral ingestion of 250-mg capsules of novobiocin (Albamycin®) or two capsules of a combination containing 250 mg of tetracycline phosphate and 125 mg of novobiocin sodium per capsule (Panalba®). Six men participated in this experiment (Table 3).

A three-way crossover experiment compared serum concentrations following the oral ingestion of novobiocin alone, tetracycline alone, or a combination. A second dose of the test drug was administered 5 hr after the initial dose. Nine subjects participated in this experiment. Doses are given in Table 4.

An exactly similar experiment in which the second dose was omitted was carried out with nine subjects.

An experiment was performed to compare serum concentrations following the oral ingestion of tetracycline phosphate (Panmycin Phosphate®), 500 mg; novobiocin sodium, 250 mg; or a combination capsule containing tetracycline phosphate, 500 mg; and novobiocin sodium, 250 mg. The test drug was given in repeated doses at 6-hr intervals for 72 hr. The nine subjects participating in the experiment took the initial dose while fasting but subsequently food and fluids were allowed as desired. All subjects adhered to a similar diet during this trial. Serum samples were collected in reference to doses 1, 5, 9, and 13.

Nine subjects participated in an experiment to compare serum concentrations following the oral ingestion of tetracycline phosphate, 500 mg; novobiocin sodium, 250 mg; or of a combination dose consisting of two 250-mg capsules of tetracycline phosphate and one 250-mg capsule of novobiocin sodium. We want to emphasize that each participant in a given experiment received each of the preparations under study.

RESULTS

The novobiocin content of the serum was consistently lower when the combination was administered than when injected alone. During the first 12 hr the values following the injection of novobiocin alone were 2 to 3½ times as high as those following injection of the combination.

Table 1. Intramuscular Novobiocin With and Without Tetracycline

Medication*	Dose, mg	Number of subjects	Average serum concentration, μg/ml, at 4-hr intervals after injection						
			0 hr	4 hr	8 hr	12 hr	16 hr	20 hr	24 hr
Novobiocin (acid)	500	5	0	4.2	8.3	8.4	7.1	7.4	5.1
Combination		5							
Novobiocin (acid)	500		0	1.2	3.2	4.4	4.5	4.7	3.9
Tetracycline hydrochloride	250		0	0.92	0.81	0.81	0.59	0.45	0.50

*85% Dimethylacetamide served as the vehicle for each preparation.

Table 2 compares the injected agents alone and in three different combinations and Table 3 concerns the single oral combination. Tetracycline concentrations during the first 12 hr were lower after each of the injected combinations than after the injection of tetracycline alone. However, tetracycline base was used in the combinations whereas tetracycline phosphate was used as the sole agent. Novobiocin concentrations were higher at 2 hr after each of the injected combinations than after novobiocin alone; these higher concentrations were sustained through 6 hr only, after injection of the combination in a dosage equal to that of the novobiocin alone. All other samples demonstrated less serum novobiocin activity after the injection of the combination of antibiotics than after novobiocin alone. However, novobiocin sodium was used in the combinations whereas novobiocin acid was used as the sole agent. In the oral combination tetracycline base was combined with novobiocin calcium. As indicated in Table 3, tetracycline levels were comparable to the injection of tetracycline phosphate but the novobiocin levels were much less than those found in any other group; of the 12 subjects only two had measurable serum novobiocin activity at 6 hr.

In the experiment comparing serum concentrations obtained with 2 : 1 and 1 : 1 oral-drop mixtures of the antibiotics, moderately good concentrations of tetracycline and relatively poor concentrations of novobiocin were found. Detailed data are not reported since comparable doses of the single antibiotics were not administered. Table 4 summarizes the results of an experiment comparing serum concentrations following the oral administration of novobiocin alone with the concentrations following the oral administration of a combination of tetracycline and novobiocin. Novobiocin serum concentrations achieved with the combination were remarkably inferior to those resulting from a dose of novobiocin alone. In this experiment novobiocin sodium was used in each preparation.

Table 5 summarizes another experiment comparing serum concentrations of the two antibiotics after their oral administration alone and in combination. Here

Table 2. Intramuscular Novobiocin and Tetracycline Alone and in Combination

Medication	Dose, mg	Number of subjects	Average serum concentration, μg/ml, at intervals after injection					
			0 hr	2 hr	6 hr	12 hr	24 hr	48 hr
Tetracycline phosphate (in 100% glycerol acetal)	250	12	0	1.80	1.60	0.99	0.51	<0.28
Novobiocin (acid) (in 85% DMA[†] in water)	500	12	0	2.12	5.25	6.95	2.92	<1.4
Combination (in 100% DMA)		12						
Tetracycline (base)	250		0	0.65	0.74	0.85	0.54	<0.28
Novobiocin sodium	500		0	8.80	9.51	4.40	2.02	<1.4
Combination (in 85% DMA in water)		12						
Tetracycline (base)	250		0	0.73	0.74	0.68	0.45	<0.80
Novobiocin sodium	250		0	2.32	1.77	(0.95)[*]	(0.36)	<1.4
Combination (in 85% DMA in diethyl carbonate)		12						
Tetracycline (base)	250		0	0.89	0.78	0.68	0.40	<0.28
Novobiocin sodium	250		0	2.76	2.05	(1.04)	(0.30)	<1.4

[*] ( ) indicates some subjects had less than minimal assayable concentrations.

[†] Dimethylacetamide.

a repeat dose of each preparation was given 5 hr after the initial dose. Tetracycline serum concentrations were lower when the combination was given than when the antibiotic was given alone: 17% less at 3 hr, 20% less at $4^{3}/_{4}$ hr, and 21% less at 8 hr. Again, however, it should be noted that tetracycline in the combinations was in the form of the phosphate whereas that given alone was the hydrochloride. Novobiocin serum concentrations were lower when the combination was given than when the antibiotic was given alone: 54% less at 3 hr, 43% less at $4^{3}/_{4}$ hr, and 29% less at 8 hr. Table 6 summarizes an experiment similar to the preceding experiment except for the omission of the second dose. Again tetracycline serum levels were less when the combination was given: 17% less at 3 hr, 22% less at $4^{3}/_{4}$ hr, and 8% less at 8 hr. Similarly, novobiocin levels were less when the combination was given: 51% less at 3 hr, 40% less at $4^{3}/_{4}$ hr, and 59% less at 8 hr.

Figures 1 and 2 summarize an experiment comparing serum concentrations of the antibiotics given alone and in combination at 6-hr intervals over a 72-hr period. Here tetracycline as the phosphate and novobiocin as the sodium were

Table 3. An Oral Liquid Combination of Novobiocin and Tetracycline

Medication	Dose, mg	Number of subjects	Average serum concentration, μg/ml, at intervals after administration					
			0 hr	2 hr	6 hr	12 hr	24 hr	48 hr
Combination (2:1)		12[*]						
Tetracycline base	500		0	2.1	2.1	1.2	0.83	<0.28
Novobiocin calcium	250		0	(0.84)[†]	(0.27)	<1.4	<1.4	<1.4

[*] These subjects also participated in the parenteral study summarized in Table 2. The oral combination served as a sixth preparation in that experiment.

[†] ( ) indicates some subjects had less than minimal assayable concentrations.

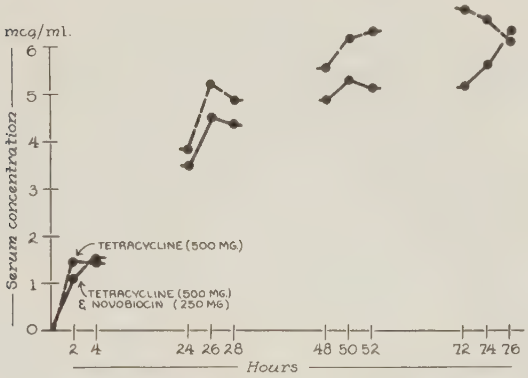

Fig. 1. Serum concentration of tetracycline following single and combined antibiotics. Oral dosages repeated every 6 hr.

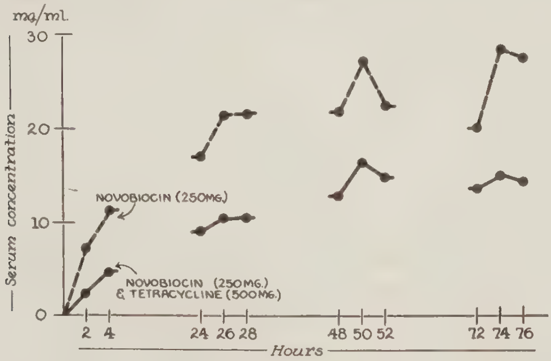

Fig. 2. Serum concentration of novobiocin following single and combined antibiotics. Oral dosages repeated every 6 hr.

used consistently. It will be noted that although the tetracycline concentrations were generally slightly less when the combination capsule was given, the values are quite comparable. In the case of novobiocin, on the other hand, lower concentrations are seen consistently with the combination; they vary from 32 to 67% less than concentrations obtained with novobiocin alone.

Table 7 summarizes another experiment comparing serum concentrations following the oral administration of the individual antibiotics with those obtained from the simultaneous administration of separate capsules containing them. In this instance the serum concentrations were comparable and in the case of novobiocin were even higher when the two capsules were administered simultaneously than when they were administered separately.

Table 4. Oral Novobiocin With and Without Tetracycline

Medication	Dose, mg	Number of subjects	Average serum concentration, μg/ml, at intervals after administration			
			0 hr	2 hr	6 hr	24 hr
Novobiocin sodium	250	6	0	7.73	3.45	<0.79
Combination		6				
Novobiocin sodium	250		0	1.74	1.64	<0.79
Tetracycline phosphate	500		0	1.51	1.57	<0.39

Table 5. Oral Novobiocin and Tetracycline Alone and in Combination With a Repeat Dose

Medication	Dose, mg	Number of subjects	Average serum concentration, μg/ml, at intervals after administration			
			0 hr	3 hr	4.75 hr	8 hr
Tetracycline hydrochloride	500	9	0	4.0	3.61	4.79 (3 hr after second dose)
Novobiocin sodium	250	9	0	11.9	7.7	9.9 (3 hr after second dose)
Combination*		9				
Tetracycline phosphate	500		0	3.33	2.90	3.79 (3 hr after second dose)
Novobiocin sodium	500		0	5.5	4.4	7.0 (3 hr after second dose)

*This medication repeated after 5 hr.

Table 6. Oral Novobiocin and Tetracycline Alone and in Combination

Medication	Dose, mg	Number of subjects	Average serum concentration, μg/ml, at intervals after administration			
			0 hr	3 hr	4.75 hr	8 hr
Tetracycline hydrochloride	500	9	0	3.27	3.33	2.26
Novobiocin sodium	250	9	0	13.0	7.5	3.4
Combination		9				
Tetracycline phosphate	500		0	2.71	2.59	2.08
Novobiocin sodium	250		0	6.4	4.5	1.4

Table 7. Oral Novobiocin and Tetracycline Given Separately and Simultaneously in Separate Capsules

Medication	Dose, mg	Number of subjects	Average serum concentration, μg/ml, at intervals after administration			
			0 hr	2 hr	4 hr	6 hr
Separate administration		9				
Tetracycline phosphate	500*		0	2.34	2.14	1.76
Novobiocin sodium	250†		0	3.72	7.41	6.83
Simultaneous administration		9				
Tetracycline phosphate	500*		0	1.91	2.38	2.08
Novobiocin sodium	250†		0	5.26	9.97	5.71

*Tetracycline dose administered as two 250-mg capsules.
†Novobiocin dose administered as one 250-mg capsule.

DISCUSSION

Although there are recognized indications for combined antibiotic therapy, it has long been the attitude of the senior author to oppose the employment of fixed combinations in a single pharmaceutical preparation.

A combination of a broad-spectrum antibiotic such as tetracycline with an effective antistaphylococcal agent such as novobiocin might be useful in the treatment of mixed infections in which both Gram-negative bacilli and staphylococci are present. Since many staphylococci have been found to be resistent to tetracycline, the addition of an effective antistaphylococcal agent seems justified. An oral preparation incorporating novobiocin has been available to the profession for some time and numerous clinical reports have appeared attesting to its effectiveness [7–13].

When it seemed possible to incorporate relatively optimal doses of tetracycline and novobiocin in a combined intramuscular preparation we elected to evaluate its activity. Primarily for this reason we made the first trial reported in this paper. The results revealed a marked inhibition of novobiocin activity when the novobiocin was administered in combination with tetracycline. In a second study the lowering of novobiocin activity proved to be more complicated and tetracycline activity seemed to be impaired.

There are certain objections to the experiment in which novobiocin was injected alone as the acid and in combination as the sodium salt. Some of the differences in serum activity may be secondary to the use of different agents. When tetracycline was injected alone, the phosphate and 100% glycerol acetal was utilized; when tetracycline was combined with novobiocin, tetracycline base and dimethylacetamide was utilized. The phosphate may be absorbed somewhat more effectively than the base but whether this explanation accounts satisfactorily for the marked difference in tetracycline serum concentrations following the injection of the two preparations is uncertain.

It is of interest also that the 1 : 1 combination resulted in considerably less than 50% novobiocin activity as compared to the 1 : 2 combination. This suggested that some binding or other interference with novobiocin occurred with the 1 : 1 combination which the excess novobiocin in the 1 : 2 combination negated. Although a combination of one part tetracycline to two parts novobiocin in 100% dimethylacetamide resulted in higher novobiocin levels, the tetracycline levels were low. The experiment, therefore, did not offer encouragement for further development of the combination.

The use of the oral-drop formulation containing novobiocin calcium and tetracycline base resulted in very low novobiocin levels, suggesting that such a formulation would have little advantage over tetracycline alone.

Our observations with oral preparations confirmed Wright's observations and those of Hirsch and Finland showing reduced novobiocin activity when novobiocin and tetracycline were combined. In fact the values obtained in our initial experiments were lower than Wright's values. We also observed small but consistently lower tetracycline serum concentrations with the oral combinations. Again, however, the use of tetracycline phosphate complex in the combinations and tetracycline hydrochloride as the single agent prohibited strict comparison of serum concentrations. When a second dose of the combination was administered at 5 hr a somewhat higher novobiocin concentration occurred 3 hr later indicating the advisability of a study of subjects taking several doses.

An experiment designed to evaluate the effects of the repeated doses of the combined versus the single drugs showed an insignificant and variable reduction in tetracycline serum concentrations following the oral ingestion of the combination, but a significant and consistent reduction in novobiocin concentrations as compared with levels obtained when the drugs were taken singly. In this experiment, tetracycline phosphate and novobiocin sodium were used in all preparations.

Several explanations might be offered for these phenomena. The presence of both agents in the gastrointestinal tract or in muscle might overload absorptive mechanisms or in some fashion interfere with the absorption of novobiocin. Variations in chemical structure and physical properties of the agents employed might account for differences in absorption. Chemical interaction between the tetracycline and novobiocin to form complexes lacking the activity of one or the other antibiotic may occur.

The results of the final experiment suggest that there is no interference with absorption from the gastrointestinal tract but the possibility of mutual interference in absorption from muscle cannot be ruled out.

Variations in chemical structure and physical properties were very likely responsible for the observed differences in some instances. For instance, in the second intramuscular study, acid novobiocin alone was compared with novobiocin sodium in the combinations. Equal doses of novobiocin sodium in the combination gave higher concentrations than acid novobiocin alone. In general the acid form of novobiocin is less soluble and less readily absorbed than the sodium salt [14]. Also, as noted previously, tetracycline phosphate alone was sometimes compared with tetracycline base in the combination. Tetracycline base is less soluble and perhaps less readily absorbed than the phosphate. On the other hand the low novobiocin serum concentrations which were apparent in all of the studies with capsular preparations cannot be attributed to differences in the physicochemical forms since the sodium salt of novobiocin was used in both single and combined drug preparations. Considering all of the data it is unlikely that the resulting differences in serum concentrations following combined therapy can be attributed entirely to differences in the physical characteristics of the compounds employed.

Since novobiocin is an acid and tetracycline a base there is a likelihood of chemical interaction between them; either the parent compounds or the salts might interact to form complexes or esters. Such reactions could readily be postulated for the solutions employed in the intramuscular preparations or in the oral-drop formulation. Unless sufficient moisture were present within the capsular forms of the combined drugs, however, it would seem more difficult to postulate a reaction of these antibiotics in the crystalline state.

The results of the final experiment, designed to test this last hypothesis, suggest the gastrointestinal mechanism for the absorption of tetracycline and novobiocin is intact and novobiocin might combine with tetracycline in such a way that it loses some of its antibiotic activity. If such a chemical reaction occurs it seems to have little effect on tetracycline activity.

ACKNOWLEDGMENTS

We especially wish to acknowledge the technical assistance of Miss Anne Rosenblatt, whose efforts were fundamental to our study. We acknowledge also the assistance and cooperation given by Harold Tucker, M.D., and Walter Sokolski, Ph.D., of The Upjohn Company. Finally, we want to express our appreciation to The Upjohn Company for the grant supporting this work and the cooperation shown us by its members.

SUMMARY

Results of experiments comparing the parenteral and oral administration of novobiocin and tetracycline alone and in various combinations indicate that novobiocin serum activity is less when it is administered in combination with tetracycline than when it is administered as the sole agent, tetracycline serum activity is slightly but not significantly less when administered in combination with novobiocin than when it is administered as the sole agent, and the simultaneous oral administration of the two agents in separate capsules results in no loss of serum antibiotic activity as compared to the oral administration of the single agents.

The data suggest novobiocin and tetracycline react to form a complex or ester lacking novobiocin activity when they are present as a mixture in either the liquid or dry state.

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DISCUSSION

DR. BONDI: I am sure the significance of the findings of these two papers is apparent to all of you. I am sure all of you will agree that many more such studies should be carried out and I wonder, Dr. Foltz, if you are carrying out any studies on any other antibiotics.

DR. FOLTZ: As yet we have not attempted any additional studies. I might say that these data were simply finished off ten days ago and we haven't had time.

DR. JACOB: May I ask what clinical significance this may have if the doctor in practice is using these combinations as they now are offered and if the blood levels achieved are high enough to do the job that is required? Is this going to be a practical obstacle to their employment?

DR. FOLTZ: I would say in reply to this that I believe that the use of such fixed combinations, at least in 95% of instances, is not justified. I think we can attribute the results to either one of the drugs alone and I would be highly suspicious that any improvement in significant staphylococcal infections that were treated by this combination was, in any sense of the word, due to the presence of the novobiocin. I think the failure to develop both adequate serum concentrations and distribution of this drug to the infected sites is a serious defect in the combinations as prepared at the present time. It's rather obvious that this can be changed in the dry-oral forms simply by keeping the materials away from each other. This is a trick that the pharmacist can perform here but I heartily subscribe to the views expressed in the recent editorial in the New England Journal in regards to the use of combinations, that in most instances they are not really indicated and the use of such fixed combinations leads to a promiscuous and perhaps indiscriminate use of effective antibiotics.

IN VITRO EVALUATION OF THE COMMONLY USED CHEMOTHERAPEUTIC AGENTS AGAINST PSEUDOMONAS ISOLATES

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INTRODUCTION

Infections caused by members of the genus Pseudomonas have received greater attention during the past few years because they are both common and troublesome. This group of organisms has been encountered more frequently in various types of clinical specimens. The distribution and frequency of isolates during the last year are shown in Table 1. They are isolated most frequently as the predominating microorganism in urine specimens and often occur in variable numbers in sputum specimens as primary and secondary agents. The data in Table 1 also illustrate their frequency in a variety of other clinical materials.

Table 1. Sources of Strains of Pseudomonas Isolated During a One-Year Period at Henry Ford Hospital

Source	Number of strains	% of total
Blood	13	4
Ear	30	9
Miscellaneous	38	12
Sputum	106	32
Throat	11	3
Urine	94	28
Wounds	39	12
	331	100

Strains of Pseudomonas are often resistant to a wide variety of chemotherapeutic agents. The experiences of many investigators indicate that this may be, at least in part, the result of the widespread use of antibiotics which inhibit other bacterial species more effectively. Therefore it is of considerable interest to determine the susceptibility patterns of these organisms in order to improve the therapeutic programs. This study includes a report of numerous tests performed with the majority of the agents currently available for the treatment of this serious clinical problem.

The study was conducted for the following reasons: (a) To determine the in vitro effect of the chemotherapeutic agents employed by all the bacteriological services. (b) To compare the susceptibility patterns of the recent strains with the 1954 isolates in order to learn whether any significant change in susceptibility patterns occurred during this six-year period. (c) To examine the records to see whether or not the isolates from certain services (i.e., urines) showed more or less resistance to certain chemotherapeutic agents (i.e., sulfonamide drugs) due to previous treatment of the patients from whom the isolates were obtained.

MATERIALS AND METHODS

Strains. Three hundred strains of Pseudomonas isolated during the past year were the primary object of this survey, but statistical data compiled since 1954 has also been utilized. The sources of the strains are listed in Table 1. There was no selection of strains; all isolates which resembled Pseudomonas were examined. The organisms were identified on the basis of macroscopic characteristics, odor of trimethylamine, and production of a metallic sheen with or without pigment production on initial isolation. The suspicious nonpigmented strains were inoculated on Mueller-Hinton (Difco), Eugon (Baltimore Biological Laboratory), or SR (Difco) media for pigment production. Additional studies such as the use of double or triple sugar-iron agar and solubility of the pigments in water and in chloroform were also done.

Susceptibility tests. The susceptibility tests were conducted using three methods [1], a) the cup-plate, b) the tube-dilution, and c) the disc procedure. The medium used for the antibiotic studies consisted of trypticase-soy agar (BBL) with 5% added sheep blood. Penassay broth and agar (Difco), Mueller-Hinton, and SR media were employed for testing the susceptibility of Pseudomonas strains to the sulfonamide compounds. The discs (Table 2) were produced commercially by both Baltimore Biological Laboratory and Difco. Frequent spot-correlation studies were performed using both brands of discs. The compounds used for tube-dilution studies were supplied by Charles Pfizer and Company and The Upjohn Company.

Table 2. Concentrations of Chemotherapeutic Agents in Discs

Agent	Low series	High series
Chloramphenicol	5 μg	30 μg
Streptomycin	2 μg	10 μg
Kanamycin	5 μg	30 μg
Neomycin	5 μg	30 μg
Nitrofurantoin	100 μg	100 μg
Nitrofurazone	100 μg	100 μg
Penicillin	2 units	10 units
Polymyxin B	50 units	300 units
Tetracycline	5 μg	30 μg
Methenamine mandelate	3.0 mg	3.0 mg
Sulfamethoxypyridazine	0.25 mg	1.0 mg
Sulfadiazine	0.25 mg	1.0 mg
Sulfisoxazole	0.25 mg	1.0 mg
Three sulfonamides	0.25 mg	1.0 mg
Sulfadiazine		
Sulfamerazine		
Sulfamethazine		

RESULTS

The majority of the strains examined produced pyocyanine or a water-soluble fluorescent pigment or both. None of the isolates produced urease or showed

Table 3. Susceptibility* of Pseudomonas Isolated from Routine Service, 1960

Agent	Number of strains tested	Sensitive, %	Intermediate, %	Resistant, %
Chloramphenicol	208	0.9	17.77	81.33
Penicillin	208	0	0.9	99.1
Tetracycline	208	0.48	7.21	92.31
Nitrofurazone	208	0.9	0.9	98.2
Polymyxin B	208	51.2	37.95	10.85
Streptomycin	208	0.9	42.28	56.82
Kanamycin	208	0.9	30.15	68.95
Neomycin	208	2.43	44.87	52.7
Sulfadiazine	100†	42.1	27.33	30.57
Sulfisoxazole	100†	12.87	40.57	46.56
Sulfamethoxypyridazine	100†	16.19	36.19	47.62
Three sulfonamides Sulfadiazine Sulfamerazine Sulfamethazine	100†	31.3	37.3	31.4

*Sensitive, inhibited by low drug concentration; intermediate, inhibited by higher drug concentration; resistant, not inhibited. See Table 2 for drug concentrations.

†Isolated on Mueller-Hinton medium.

fermentation on triple sugar-iron agar in 48 hr. The most reliable test for production of pigments was the formation of a blue or green color on Eugon or Mueller-Hinton agar. All strains that produced such reactions were identified as members of the genus Pseudomonas.

The data for the susceptibility tests of more than 300 Pseudomonas strains isolated during 1960 illustrate the susceptibility patterns of isolates obtained from two of our bacteriological services, a) routine service, i.e., (throats, sputums, wounds), and b) urine service. The information is tabulated according

Table 4. Susceptibility* of Pseudomonas Strains Isolated from Urines, 1960

Agent	Number of strains tested	Sensitive, %	Intermediate, %	Resistant, %
Chloramphenicol	104	3.03	16.11	80.86
Penicillin	104	0	2.02	97.98
Tetracycline	104	0	13.46	86.99
Nitrofurantoin	104	0.9	2.9	96.2
Polymyxin B	104	59	29	12
Streptomycin	104	1.04	37.12	60.84
Kanamycin	104	1.02	26.5	72.48
Neomycin	104	1.07	37.6	61.33
Sulfisoxazole	104	3.61	8.42	87.97
Sulfamethoxypyridazine	104	1.2	3.6	95.2
Methenamine mandelate	104	21.3	62.9	15.8
Three sulfonamides Sulfadiazine Sulfamerazine Sulfamethazine	104	8.6	21.6	69.8

*Sensitive, inhibited by low drug concentration; intermediate, inhibited by higher drug concentration; resistant, not inhibited. See Table 2 for drug concentrations.

to the chemotherapeutic agents which were requested most frequently by the clinicians at Henry Ford Hospital. Since these studies vary not only with the bacteriological service but also with the type of specimen and the special requests of the clinician, the agents listed for each service may be slightly different. For example nitrofurazone, a topical preparation, would be requested for burn cases but not for systemic use.

The variations demonstrated by the various techniques (cup-plate, tube-dilution and disc) employed for sensitivity testing have been discussed by Jackson et al. [2] and Truant [3]. The tables presented here deal with the results obtained by the disk procedure. It should be pointed out that discrepancies due to methods do occur but the results are generally comparable.

The data show clearly that polymyxin B was by far the most active of this group followed by neomycin, streptomycin, and kanamycin. Table 3 also shows that the sulfa drugs have considerable activity against over 50% of the strains isolated from the routine service but considerably less activity against the isolates from urine (Table 4). This can be explained in part by the fact that a large majority of the isolates from urine specimens were obtained from patients previously treated with these compounds. This explanation has been made on the basis of studies conducted in urology patients who were frequently treated conventionally with sulfa drugs (i.e., sulfisoxazole). If the infection was resistant to treatment the urine was submitted for culturing and susceptibility testing of the isolates.

Table 5 reveals the differences in susceptibility patterns based on the use of two different media, Mueller-Hinton and SR. The data clearly illustrate that a higher percentage of organisms show greater susceptibility on SR than on Mueller-Hinton. The results also demonstrate considerable activity by these sulfonamides against the isolates obtained on the routine service.

Table 5. Susceptibility of Pseudomonas Strains (208) Isolated from Routine Service, 1960, on Mueller-Hinton or SR Medium

Susceptibility	Sulfadiazine		Sulfisoxazole		Sulfamethoxypyridazine		Three sulfonamides*	
	M-H, %	SR, %	M-H, %	SR, %	M-H, %	SR, %	M-H, %	SR, %
Sensitive	42.1	50.0	12.87	42.9	16.19	41.42	31.3	37.5
Intermediate	27.33	40.0	40.57	28.58	36.19	14.28	37.3	50.0
Resistant	30.57	10.0	46.56	28.52	47.62	44.30	31.4	12.5

*Sulfadiazine, sulfamerizine, sulfamethazine

A summary of all strains isolated on the two bacteriological services shows clearly the in vitro superiority of polymyxin B and also demonstrates that such antibiotics as neomycin, streptomycin, kanamycin, chloramphenicol, and tetracycline might also be considered seriously by the clinician. The data reveal the great variation in susceptibility to the sulfonamides between the more resistant isolates from urine and the more sensitive strains isolated from the routine service.

For comparison, Table 6 lists the results obtained with Pseudomonas strains isolated in 1954 and 1960. A survey of the data shows no significant difference for polymyxin B but does show what appears to be a significant increase in the number of resistant strains for chloromycetin, streptomycin, and tetracycline during the six-year interval.

Table 6. Comparison of Susceptibility Patterns for Pseudomonas Strains Isolated in 1954 and 1960

Susceptibility	Polymyxin B		Streptomycin		Chloramphenicol		Tetracycline	
	1954, %	1960, %	1954, %	1960, %	1954, %	1960, %	1954, %	1960, %
Sensitive	87.5	88.5	75.0	41.0	31.2	19.0	31.3	10.0
Resistant	12.5	11.5	25.0	59.0	68.8	81.0	68.7	90.0

DISCUSSION

There is no complete agreement on the criteria for the identification of Pseudomonas strains. The identification of various types of organisms in this group is difficult since a wide variety of species is included in the genus as shown by Breed et al. [4]. Although pigment production is not entirely reliable it is the most striking characteristic and therefore has been used to identify isolates belonging to this group. The ability of different strains to produce pigment may be demonstrable only on certain media. It has been our experience that better pigment production is obtained on Eugon and Mueller-Hinton agar rather than on SR or blood-agar media. Wright et al. [5] employed Gessard's glycerol agar to demonstrate pigment production but we have had no experience with this medium. The strains which did not show pigment production but which produced the marked odor of trimethylamine and a typical reaction on Kligler's or Russel's sugar-iron media were examined for pyocyanine production by extraction from an alkaline media with chloroform resulting in the appearance of a deep blue pigment. Wright et al. [5] have discussed further tests which may be of importance in the identification of Pseudomonas strains.

The susceptibility tests shown in Tables 3 and 4 indicate that chloramphenicol is the most effective of the first four compounds but inhibits less than 20% of the strains. Tables 3 and 4 illustrate the marked superiority of polymyxin B among the chemotherapeutic agents tested in vitro against the majority of the more than 300 strains of Pseudomonas. More than 50% of the strains were susceptible to polymyxin B. Neomycin and streptomycin were active against 40 to 50% of the isolates but were considerably less effective against many strains which were susceptible to polymyxin B. The group of antibiotics shown in Table 3, though showing marked in vitro activity, produce marked side effects when used systemically. Therefore their in vivo use is based on the clinician's critical evaluation of the seriousness of the infection, clinical history, and other considerations.

It is interesting to note that the sulfonamides demonstrated considerable in vitro activity against many of the organisms and this group of therapeutic agents might well be considered for the treatment of infections due to Pseudomonas.

It should be emphasized that both the laboratory technician and the clinician should be aware of the antagonists which may be present in both media and inoculum, as shown by Havas and McGeady [6]. The data in Table 5 demonstrate some of the variations resulting from the use of different media. Experience in our laboratory has shown that the size of the inoculum is also a factor to be considered in testing for sulfonamide activity.

A summary of the susceptibility patterns for all the strains tested with various antibiotics reveals that polymyxin B possesses greater activity than any other antibiotics listed. It should be pointed out that there was a marked difference between the susceptibility patterns of organisms from the urine and those isolated from the routine service. The difference is due possibly to the large number of urine specimens which are obtained from patients who have previously been treated unsuccessfully with sulfonamides.

Table 6 shows that a marked increase occurred in the resistance to all antibiotics except polymyxin B since 1954. This has also been the experience of other investigators.

SUMMARY AND CONCLUSIONS

Strains of Pseudomonas isolated during the past year were tested for susceptibility to the commonly-used antibiotics and sulfonamides. Similar studies have been conducted during the past six years and have been discussed briefly. Polymyxin B was by far the most active agent in vitro. However, the following agents showed slight to moderate activity: neomycin, streptomycin, kanamycin, the sulfonamides, and chloramphenicol. The 312 strains of Pseudomonas were usually resistant to penicillin.

It is important to consider the sulfonamides in testing for in vitro activity since a significant percentage of Pseudomonas are susceptible. The methods used for testing sulfonamide activity are more selective than those employed for antibiotic susceptibility testing.

A comparison of the data obtained between 1954 and 1960 shows a significant increase in the percentage of Pseudomonas resistant to chloramphenicol, streptomycin, and tetracycline but not in those resistant to polymyxin B.

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DISCUSSION

Q: I would like to comment on the fact that we did a similar study over a five-year period ending in 1957 at Los Angeles Children's Hospital in which our results were almost identical to those of Dr. Truant. This was reported in the New England Journal just a few months ago. The striking thing at that time was the marked reduction in sensitivity over the period of time in which we began to use more chloramphenicol with the Pseudomonas organism. The sensitivity pattern changed from approximately 30% sensitive to approximately 8% sensitive in a period of one year when chlorampehnicol was used more extensively. We again repeated our studies over the past six months on an outpatient basis and part of this was reported this morning on the studies on Declomycin. The results were very comparable except that there was somewhat more sensitivity to chloramphenicol than we had found in our previous study. Our figures were, approximately, 8% sensitive to tetracycline, 28% to chloramphenicol, 60% to streptomycin, 33% to neomycin, and again, as reported, 90% to polymyxin B. This 90% has been a very consistent figure in every study that we have done. Again we found the same thing that Dr. Truant did, that a high percentage of the organisms were sensitive in vitro to mandelamine. Our conclusions on the study of Pseudomonas infections in the 1957 study was that serious infections could best be treated with combined therapy using polymyxin B as the basic drug and adding either terramycin or streptomycin to this treatment. Oxytetracycline was found generally to be preferable to tetracycline in that more strains were sensitive to it.

Q: I would like to know whether you use the procedure routinely? Are your results based on the disc test or tube-dilution test? The point I want to make here is that there has been a marked improvement in the preparation of the discs.

DR. BONDI: Dr. Truant, using the different test methods there was some discrepancy. Is this more true with certain agents than others?

DR. TRUANT: This goes back to the hassle we had here some two years ago. The percentage will vary between zero and 15% and there is no doubt that polymyxin B, which diffuses more slowly, does give greater variation under certain conditions. So you have to evaluate the methods critically. More important, I should remind the group that one important factor we have learned is that the inoculum size in sulfonamide testing is rather critical.

DR. SCHENK: What levels in your tube-dilutions did you take as resistant or sensitive in correlation with the levels you obtained in vivo?

DR. TRUANT: These are listed in the text in detail. For the mycins it runs along the standard pattern of 5 through 30 μg. With things like penicillin, of course, it is 2 to 10 μg with furacin, and furadantin, it is 100 μg and it goes all the way along the line using the standard concentration range. These are listed in the text.

DR. BONDI: I was hoping one of the clinicians might like to comment on the in vitro in vivo correlation in treatment of Pseudomonas infections. As an enthusiast for laboratory testing as a guide to therapy, I have felt that there has always been a relatively good correlation between results obtained in the laboratory and those obtained in therapy. However, there has always been a little doubt in my mind as to how good the correlation is in reference to Pseudomonas. Now I am not inferring that Pseudomonas infections or strains of Pseudomonas resistant to penicillin and tetracycline are going to respond to these agents. But I wonder if there are times when some of the Pseudomonas strains are sensitive to a few of the agents in the laboratory, where the correlation is still not very good, and the therapeutic results leave very much to be desired.

DR. TRUANT: I won't challenge this but I will make one statement to remind everyone that the clinician has to look at this from a large number of standpoints and certainly it is a very critical problem. I want you to know that these are in vitro results and that this, of course, is chiefly a clinical decision.

THE EFFECT OF NITROFURANS ON THE SURVIVAL OF BACTERIA IN VIVO

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INTRODUCTION

A critical consideration in antimicrobial therapy is the necessity of maintaining a drug concentration in contact with microorganisms long enough to result in sterilization. Because the drug is administered intermittently and is absorbed and excreted irregularly, the levels constantly fluctuate at the site of infection. This interaction of alternating drug levels on different cell populations is difficult to assess not only in experimental situations where complicating host defense mechanisms interplay with the drug—parasite relationship but also in the establishment of an in vitro model for the analysis of this interaction. In the consideration of these aspects it was observed that the efficacy of various nitrofurans was not reflected in appreciably high blood levels. Indeed, this apparent paradox of low blood levels and efficacy has been difficult to reconcile in view of the amounts necessary to demonstrate activity by tube-dilution or disc methods. It is recognized, however, that the variables which influence these methods do not necessarily duplicate comparable in vivo situations and in many instances do not lend themselves to analogous interpretation.

The purpose of this report therefore, is to present studies of the action of furaltadone* [N-(5-nitro-2-furfurylidene)-3-amino-5-(N'-morpholinylmethyl)-2-oxazolidone] on Staphylococcus aureus in experimental animals. In some comparative experiments the action of furazolidone† [N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone] on Salmonella typhosa is included. In addition, bacterial population changes which occur in cellophane dialysis sacks in vivo are correlated with in vitro findings.

MATERIALS AND METHODS

Cultures. S. aureus strain Mi-6, a hemolytic, coagulase-positive organism and S. typhosa strain Ty-2, a motile, Vi-positive organism were used. These cultures were grown on brain-heart infusion agar (BHIA) for 18 hr at 37 C. The optical densities of these cells, suspended in 0.85% NaCl, were determined spectrophotometrically and correlated with a reference optical density vs viable-cell graph.

Animals. CFW white male mice weighing 18-20 g were used.

Drug concentration. Furaltadone and furazolidone concentrations in plasma and in aqueous solutions were determined by methods reported previously [1, 2]. Drugs were administered orally in 0.75% carboxymethylcellulose in 0.5-ml volumes.

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*The trade name of Eaton Laboratories for furaltadone is Altafur.

†The trade name of Eaton Laboratories for furazolidone is Furoxone.

Cellophane dialysis sacks. Cellophane tubing (Visking Corp.) was cut in 10-cm lengths and tied at one end by suture. Cotton-plugged glass tubings were inserted into the open ends and the tubings were autoclaved in water. Saline suspensions of bacteria were inoculated and the open ends tied off. These cellophane sacks were inserted through small intraperitoneal incisions in anesthetized animals. The incisions were closed with suture clamps.

EXPERIMENTAL

In vivo studies. S. aureus cells suspended in 5% mucin were injected intraperitoneally (i.p.) into groups of five mice each. These inocula represented lethal doses ($1 \cdot 10^7$ cells) and maximum nonlethal doses ($1 \cdot 10^6$ cells). Furaltadone was administered at 100 mg/kg and 25 mg/kg immediately following the bacterial challenge (0 hr). In some reported instances the drug at 25 mg/kg was given at 0 and 4 hr and at 0, 4, and 8 hr after challenge. Viable counts were made by washing the peritoneal cavities with saline and plating on BHIA. The results of these experiments are assembled in Figs. 1 and 2 to permit comparison of the effect of varying regimens of drug administration on different challenge inocula.

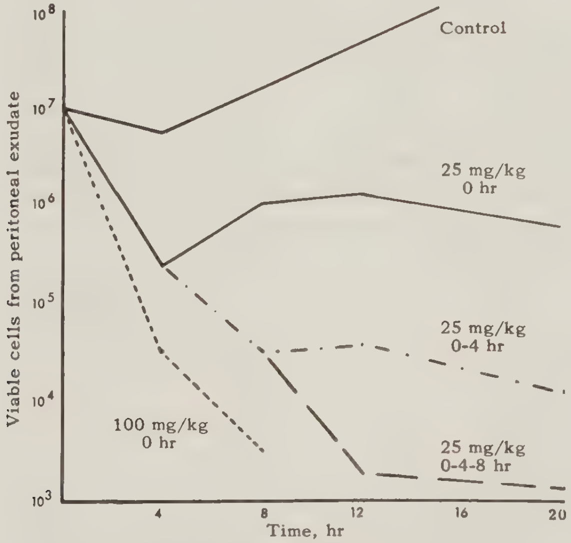

Fig. 1. The effect of single and multiple doses of furaltadone upon lethal challenges of Staphylococcus aureus.

These data show a rapid and progressive decline in viable organisms in the presence of furaltadone. This decline was dependent upon the amount and frequency of drug administration. Activity of 4 hr duration was noted when the drug was given at 25 mg/kg whereas somewhat longer activity was obtained at 100 mg/kg. Approximately a tenfold decrease in viable cells occurred following each successive dose of 25 mg/kg. A single dose of 100 mg/kg was comparable to three doses of 25 mg/kg although a shorter time was required to reach this thousandfold loss of viability with the single dose. Furaltadone activity was equally effective against S. aureus in mice receiving lethal and maximum nonlethal challenges.

From the results presented above it appeared that successive losses to bacterial populations followed repeated administration of furaltadone. It was of interest therefore, to determine the activity of another nitrofuran, furazolidone,

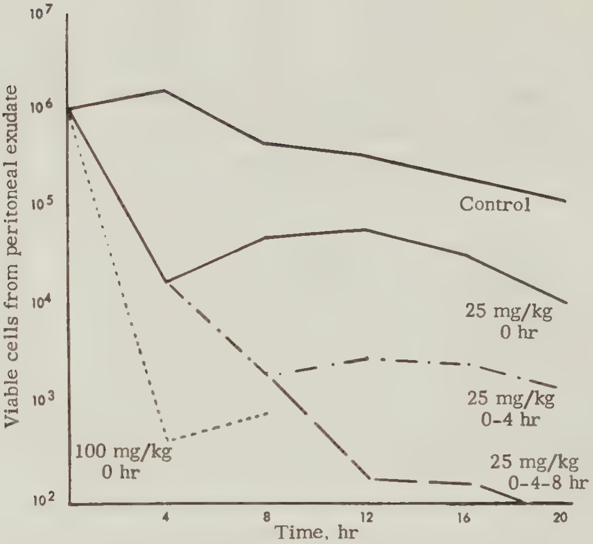

Fig. 2. The effect of single and multiple doses of furaltadone upon maximum nonlethal challenges of Staphylococcus aureus.

against S. typhosa in mice. S. typhosa cells suspended in saline were inoculated i.p. with $1 \cdot 10^8$ cells (LD_{100}) and with $8 \cdot 10^6$ cells (LD_0). Furazolidone was administered at 25 mg/kg according to the procedure used for furaltadone. In Fig. 3 are seen the effects of 1, 2, and 3 doses of lethal and sublethal challenges. Each 25 mg/kg dose initiated a ten- to fifteenfold decrease in viable cells during a

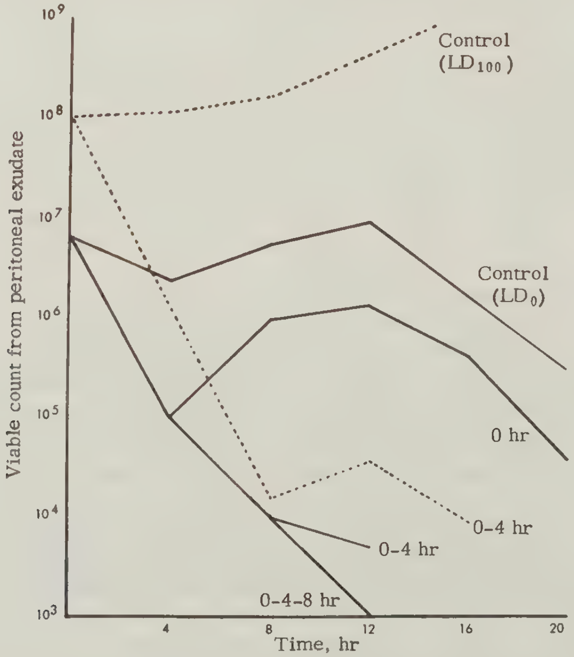

Fig. 3. The effect of single and multiple doses (25 mg/kg) of furazolidone upon lethal and maximum nonlethal challenges of Salmonella typhosa.

4-hr period. These data were comparable to those obtained with furaltadone against *S. aureus* not only in duration of activity but also on the rate of bacterial decline.

It may be well to point out here that although a single 25-mg/kg dose of nitrofuran was sufficient to reduce the viable cells below lethal numbers, these studies demonstrated a rapid elimination of surviving organisms upon additional treatment.

Nitrofuran concentrations. In order to assess more closely the relationship between in vivo drug concentrations and the demonstrated efficacy of nitrofurans, an analysis of furaltadone and furazolidone levels in mice was made. Groups of 15 mice each were fed these nitrofurans at 100 mg/kg and 25 mg/kg. At 1, 2, and 4 hr, groups were exsanguinated and the drug concentrations determined on the pooled plasma. Within 1 hr both nitrofurans reached peak levels of approximately 6 to 8 μg/ml when administered at 100 mg/kg. By the fourth hour 50% of the initial concentrations were present. Plasma from mice receiving 25 mg/kg also reached peak levels of 3 to 4 μg/ml within 1 hr with a concomitant decline of 50% at 4 hr. No significant difference in plasma levels was found between furaltadone and furazolidone.

In conjunction with these studies, cellophane dialysis sacks containing water were inserted i.p. in mice immediately following the administration of the nitrofurans at 100 mg/kg. The cellophane sacks were removed at 1, 2, and 4 hr and the concentrations assayed from the water samples. Comparable peak levels of 8 μg/ml of furaltadone and of furazolidone were reached within 1 hr. The levels were approximately 3 μg/ml in 4 hr. The levels of nitrofurans which had diffused into these sacks paralleled levels obtained in blood plasma.

Table 1. The In Vitro Sensitivity of *Staphylococcus aureus* and *Salmonella typhosa* to Furaltadone and Furazolidone

Organism vs drug	Number of cells/ml in broth	Drug concentration, μg/ml	Viable count			
			2 hr	4 hr	6 hr	8 hr
S. aureus vs Furaltadone	10^6	10	$1 \cdot 10^4$	0	–	–
		6	$8 \cdot 10^4$	$5 \cdot 10^3$	0	–
		2	$6 \cdot 10^5$	$5 \cdot 10^5$	$7 \cdot 10^5$	$2 \cdot 10^7$*
	10^7	10	$1 \cdot 10^5$	$2 \cdot 10^4$	$1 \cdot 10^4$	$8 \cdot 10^2$
		6	$1 \cdot 10^6$	$3 \cdot 10^5$	$2 \cdot 10^4$	$1 \cdot 10^3$
		2	$7 \cdot 10^6$	$8 \cdot 10^6$	$4 \cdot 10^7$	$6 \cdot 10^8$*
	10^8	10	$2 \cdot 10^6$	$5 \cdot 10^5$	$5 \cdot 10^5$*	
		6	$2 \cdot 10^7$	$2 \cdot 10^7$	$2 \cdot 10^7$*	
		2	$2 \cdot 10^8$	$4 \cdot 10^8$	$5 \cdot 10^8$*	
S. typhosa vs Furazolidone	10^6	10	$1 \cdot 10^3$	0	–	
		6	$1 \cdot 10^4$	$2 \cdot 10^3$	0	
		2	$6 \cdot 10^4$	$3 \cdot 10^4$	0	
	10^7	10	$1 \cdot 10^3$	$1 \cdot 10^2$	0	
		6	$2 \cdot 10^5$	$2 \cdot 10^3$	0	
		2	$8 \cdot 10^5$	$6 \cdot 10^5$	$6 \cdot 10^6$*	
	10^8	10	$3 \cdot 10^5$	$2 \cdot 10^5$	$1 \cdot 10^6$*	
		6	$4 \cdot 10^6$	$4 \cdot 10^6$	$4 \cdot 10^6$*	
		2	$3 \cdot 10^7$	$6 \cdot 10^7$	$1 \cdot 10^8$*	

*Visible growth in 24 hr. Growth in control was similar to that of controls in Table 2.

In vitro sensitivity. *S. aureus* and *S. typhosa* organisms were inoculated into BHI broth containing furaltadone and furazolidone, respectively. The cultures contained $1 \cdot 10^8$, $1 \cdot 10^7$, and $1 \cdot 10^6$ cells/ml in drug concentrations of 10, 6, and 2 μg/ml. After incubation at 37 C for 2, 4, 6, and 8 hr, the surviving bacteria were assayed by plating on BHIA. The data in Table 1 show that nitrofuran activity was dependent upon inoculum size. A tenfold decrease in cells required lower drug concentrations to reduce viability whereas the rate of decline was accelerated with higher concentrations. Furaltadone activity against *S. aureus* was slightly less effective than furazolidone against *S. typhosa* during comparable time periods. However, this difference was not reflected upon further incubation for 24 hr. At concentrations less than bactericidal the nitrofurans exerted bacteriostatic activity of 2-6 hr duration. In addition, the results compared favorably with the in vivo data on the obtainable drug plasma levels following the administration of nitrofurans. Previous studies [3] have demonstrated the effect of inoculum size on the antibacterial activity of furaltadone at 24 and 48 hr.

Table 2. The In Vivo Sensitivity of *Staphylococcus aureus* and *Salmonella typhosa* in Cellophane Sacks in the Peritoneal Cavity of Mice to 100 mg/kg of Nitrofurans

Organism vs drug	Number of cells	Viable count		
		2 hr	4 hr	6 hr
S. aureus vs Furaltadone	10^6	$2 \cdot 10^5$	$1 \cdot 10^3$	0
	10^7	$1 \cdot 10^6$	$2 \cdot 10^5$	$1 \cdot 10^2$
	10^8	$2 \cdot 10^7$	$8 \cdot 10^6$	$1 \cdot 10^6$
S. aureus control	10^6	$2 \cdot 10^6$	$2 \cdot 10^7$	$9 \cdot 10^7$
	10^7	$3 \cdot 10^7$	$2 \cdot 10^8$	$1 \cdot 10^9$
	10^8	$8 \cdot 10^7$	$1 \cdot 10^9$	$2 \cdot 10^9$
S. typhosa vs Furazolidone	10^6	$2 \cdot 10^5$	$3 \cdot 10^4$	0
	10^7	$1 \cdot 10^6$	$1 \cdot 10^5$	0
	10^8	$1 \cdot 10^7$	$4 \cdot 10^5$	$2 \cdot 10^3$
S. typhosa control	10^6	$2 \cdot 10^6$	$2 \cdot 10^7$	$2 \cdot 10^8$
	10^7	$2 \cdot 10^7$	$3 \cdot 10^8$	$7 \cdot 10^8$
	10^8	$2 \cdot 10^8$	$5 \cdot 10^8$	$2 \cdot 10^9$

In order to determine the rate of furaltadone depletion in the presence of *S. aureus*, cultures of $1 \cdot 10^8$ and $1 \cdot 10^7$ cells were added to broth containing 10 g/ml of drug. At 2 and 3 hr, aliquots of the cultures were centrifuged and the supernates analyzed for furaltadone. The results showed that cultures containing $1 \cdot 10^8$ cells reduced the nitrofuran level by 40% at 2 hr and by 60% at 3 hr. Cultures of $1 \cdot 10^7$ cells had a drug loss of 20% in 2 hr and of 30% in 3 hr. The initial concentration was depleted at a rate dependent upon the inoculum size.

Cellophane sacks in vivo. Studies were made to determine quantitatively the drug—parasite relationship while excluding complex host factors. Cellophane sacks containing *S. aureus* and *S. typhosa* in water suspensions were inserted i.p. into mice immediately following the administration of 100 mg/kg of nitrofurans. The sacks were removed at 2, 4, and 6 hr and assayed for viable cells. The results are given in Table 2 and show that a rapid loss in viability was obtained following the diffusion of the drugs into the sacks. Bactericidal action was accelerated with smaller cell numbers. Controls were not affected by any adverse diffusable substance and multiplied at a rate indicating a rapid diffusion of nutrients. Furazolidone was slightly more effective against *S. typhosa* than was furaltadone against *S. aureus*. However, these nitrofurans were highly effective in reducing cell viability. This drug—parasite interaction in mice was comparable to the in vitro sensitivity of these organisms to concentrations of 6 and 10 μg of nitrofurans (Table 1). Furthermore, drug plasma levels of 6 to 8 μg, obtained upon the administration of 100 mg/kg, correlated with the loss of cell viability in the in vivo studies.

DISCUSSION

The present work showed that the administration of a single dose of nitrofurans produced major alterations in the viability of lethal and maximum nonlethal challenges of microorganisms. This effect reduced the bacterial populations to levels capable of being reduced further by the host—parasite interaction. Each succeeding dose of furaltadone and furazolidone accelerated the decline of *S. aureus* and *S. typhosa* cells. The drug action at 25 mg/kg persisted for periods of 4 hr whereas with higher doses a longer period of activity was obtained. This 4-hr activity was related to mouse-plasma drug levels which showed peak plasma levels in 1 hr with a gradual reduction to 50% in 4 hr.

The in vitro sensitivity tests demonstrated considerable nitrofuran activity upon *S. aureus* and *S. typhosa*. An acceleration of susceptibility was dependent not only upon drug concentration but also upon inoculum size. Within the plasma levels of 6-8 μg and 3-4 μg following drug administration of 100 mg/kg and 25 mg/kg, comparable in vitro concentrations exerted marked bactericidal action on large numbers of organisms. This activity was rapid and compared favorably with in vivo results. The depletion of nitrofurans from the in vitro environment correlated with bactericidal action. Another point on which this finding has some bearing is the apparent in vitro insusceptibility of organisms to low drug levels when the observations are based on visible growth at 24 hr. The growth of organisms not adversely affected before the drug level diminished would consequently overshadow any therapeutic activity. However, the data presented here on low drug concentrations during the first 6 hr of contact with microorganisms demonstrated marked bactericidal activity. The bactericidal action of these drugs on *S. aureus* and *S. typhosa* cells contained in cellophane dialysis sacks further indicated that in the in vivo situation the over-all plasma drug levels tended to maintain constant levels during a steady rate of decline and were not subject to the same variables present in a closed in vitro environment.

SUMMARY

Viable counts from mouse peritoneal exudates showed a rapid and progressive decline of viable *Staphylococcus aureus* and *Salmonella typhosa* cells following the administration of single and multiple doses of furaltadone and furazolidone. The rate of cell decline was dependent upon inoculum size and the amount of nitrofuran administered. In vitro sensitivity tests comparable to mouse-plasma drug levels demonstrated marked bactericidal activity on these organisms during the first hours of contact. Evidence is presented which shows that nitrofurans were rapidly depleted from the in vitro environment and that the depletion correlated with bactericidal activity. A technique using cellophane dialysis sacks in vivo for the analysis of drug—parasite interactions is described.

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SPORICIDAL ACTIVITIES OF A NUMBER OF ALDEHYDES

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Paper withdrawn at the request of the authors

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THE ELEVATED ANTISTREPTOLYSIN O TITER OF POOLED HUMAN GAMMA GLOBULIN

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INTRODUCTION

Pooled normal human gamma globulin has been reported by Fisher and Manning [1] to exert therapeutic activity in experimental Streptococcus pyogenes infections of animals; marked potentiation of antibiotic activity by pooled gamma globulin in treatment of such infections has been described by Fisher [2]. In seeking to understand the mechanism of this protection, we decided to investigate the effects of pooled gamma globulin on the various streptococcal components and metabolites implicated in virulence. The oxygen-labile exotoxin, streptolysin O, was the streptococcal factor selected for this pilot study for the following reasons: (a) Streptolysin O has been implicated in the pathogenesis of disease caused by the organisms while the antibody, antistreptolysin O, has been shown to afford protection against certain of the toxic effects of the metabolite. Thus, the presence of this antibody in pooled gamma globulin might afford a partial explanation of the observed therapeutic activity of the concentrated serum product. (b) The finding in pooled human gamma globulin of antistreptolysin O might serve as a convenient index to the presence in gamma globulin of antibodies to other streptococcal factors implicated in the in vivo expression of virulence by these organisms. (c) Standardized materials for the in vitro determination of antistreptolysin O serum levels have become readily available for use in a universally accepted titration procedure. (d) Information on the antistreptolysin O serum titers of normal human populations and persons ill with streptococcal disease is available for study for the determination of the efficacy wherewith this antibody has been concentrated in commercial pooled gamma globulin.

The properties of streptolysin O have been reviewed comprehensively by Bernheimer [3]. This exotoxin is formed by streptococcal strains belonging to Lancefield's Groups A, C (human), and large colony G in vitro under conditions of suitably reduced oxygen-reduction potential and in naturally occurring infections in man. The lysin is activated by sulfhydryl groups, becoming nonhemolytic on standing in the presence of oxygen. Cholesterol readily neutralizes the hemolysin in the test tube; none of the cholesterol of normal serum, however, is in a state in which it can combine with the hemolysin. Preparations of streptolysin O exhibit typical protein properties, being thermolabile, readily destroyed by proteolytic enzymes, and nondialyzable. Streptolysin O is antigenic and antisera, potent in the inhibition of erythrocyte lysis, are formed in humans, horses, and rabbits following infection with streptococci producing this toxin. Oxidized as well as reduced streptolysin O combines with the specific antibody, antistreptolysin O.

This investigation has indicated that pooled normal human gamma globulin has a very high antistreptolysin O content, approximately 20 to 30 times the average serum-antibody titer seen in surveys of normal adult human populations.

MATERIALS AND METHODS

Gamma globulin in preparations. Seven lots of poliomyelitis-immune globulin, the products of six different commercial manufacturers, were studied. Each product had been processed from large pools of adult human blood and contained 165 (±1.5) mg gamma globulin/ml. One preparation[*] free of preservative was available for experimental purposes. The six commercial preparations[†] each contained sodium ethylmercurithiosalicylate in a final concentration of 1 : 10,000.

Antistreptolysin O titration. Levels of antistreptolysin O in each pooled gamma globulin preparation were titrated by the Rantz and Randall [4] modification of the standard procedure of Todd [5] using a single lot of Bacto-Streptolysin O Reagent (Difco). This hemolysin had been standardized against the primary antistreptolysin O standard of Todd, a preparation of concentrated horse-serum globulins containing 20,000 antistreptolysin O units/ml. Thus, in the assay of the antistreptolysin O titer of pooled gamma globulin, the capacity of this product to neutralize the hemolytic activity of a test dose of streptolysin O was ultimately compared to that of the standard Todd serum.

The test as performed was a serial tube-dilution procedure measuring the hemolytic capacity of a fixed constant concentration of streptolysin O in the presence of gamma globulin assayed at various concentration levels. The hemolysis indicator was erythrocytes from albino New Zealand rabbits. Gamma globulin was titrated at final dilutions of 1 : 12, 1 : 50, 1 : 100, 1 : 125, 1 : 166, 1 : 250, 1 : 333, 1 : 500, 1 : 625, 1 : 833, 1 : 1250, 1 : 1666, 1 : 2500, and 1 : 5000. The antistreptolysin O titer expressed in Todd units was the reciprocal of the highest dilution of gamma globulin showing no hemolysis.

Table 1. Antistreptolysin O Titers of Seven Pooled Human Gamma Globulin Preparations

Gamma globulin preparation	Titer, Todd units
A[†]	1666
B[†]	1666
C[†]	2500
D[*]	1666
E[†]	2500
F[†]	1250
G[†]	1666

[*] Poliomyelitis-immune globulin (human) (Lot No. C-583) prepared by Lederle Laboratories Division, American Cyanamid Co. This preparation contained no preservative.

[†] Poliomyelitis-immune globulin (human) preparations of six different commercial manufacturers. The preservative in each preparation was sodium ethylmercurithiosalicylate (1 : 10,000).

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[*] Poliomyelitis-immune globulin (human) (Lot No. C-583) prepared by Lederle Laboratories Division, American Cyanamid Co., New York City.

[†] Poliomyelitis-immune globulin (human) prepared by: Cutter Laboratories, Berkeley, California (Lot No. G0370); Hyland Laboratories, Los Angeles (Lot No. 441F11B); Lederle Laboratories Division, American Cyanamid Co. (Lot No. 2175-104A7300); Merck Sharp & Dohme Division, Merck & Co., Inc., Philadelphia (Lot No. 046081B); and Pitman Moore Division of Allied Laboratories, Indianapolis, Indiana (Lot No. 46948B-1).

RESULTS AND COMMENT

Table 1 indicates the high antistreptolysin O potency found for the seven preparations of pooled gamma globulin studied. It can be seen that antistreptolysin O titers ranged from 1250 to 2500 Todd units.

A comparison of the antistreptolysin O titer levels of pooled gamma globulin with the average serum levels observed in healthy persons and in patients either suffering from acute streptococcal disease or convalescent from clinical infection is important. This permits a better comprehension of the extent to which pooled globulin has concentrated antibody from large pools of blood representing the combined blood samples from thousands of adults. The comparison data are presented in Table 2.

Table 2. Average Serum Antistreptolysin O Titers of Various Population Groups as Compared to the Titers of Preparations of Pooled Human Gamma Globulin

Population group	Titer, Todd units
Children <3 years of age*	<12
Children 5-12 years of age*	238
Military personnel*	117
Adults >40 years of age*	33
Children 6-8 years of age†	100
Adults: Miami residents†	75
Adults: Airline personnel†	71
Acute streptococcal pharyngitis in children 6-8 years of age‡	250-625
Acute rheumatic fever§	405
Acute rheumatic fever‖	±500
Pooled gamma globulin#	1250-2500

*Rantz, et al. [6]
†Streitfeld and Saslaw [7,8]
‡Streitfeld and Saslaw [9]
§Rantz [10]
‖McCarty [11]
#Poliomyelitis-immune globulin (human) of six different commercial manufacturers.

Average antistreptolysin O titers in different age groups have been found to differ considerably. Rantz et al. [6], in studies of healthy subjects in California found that children under 3 years of age had an average serum titer of less than 12 Todd units; an average titer of 238 was observed for children 5 to 12 years of age, while healthy military personnel were seen to have an average titer of 117 and adults over 40 years of age had the relatively low average titer of 33. In studies by Streitfeld and Saslaw [7, 8] in Florida, assays of 996 serum samples from clinically well children yielded an average titer of 100. Assays of 796 serum samples from healthy adults who were residents in the area gave an average titer of 75. An average titer of 71 was obtained from serum samples from 816 healthy airline employees reporting from positions throughout the Eastern seaboard for routine medical examination in the airline's central medical clinic in Miami.* We have found these titers to reflect the following facts: (a) In a civilian population under nonepidemic conditions school children suffer a greater number of infections with Group A beta-hemolytic streptococci than do adults, the infection rates for Lancefield's Groups C and G in children and adults being approximately equal. (b) School children 6 to 8 years of age are exposed to a greater number of clinical and subclinical infections with beta-hemolytic streptococci, while not yet having acquired immunity to the prevalent Lancefield types of Group A beta-hemolytic streptococci.

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*Eastern Airlines, Inc.

Average serum titers in patients suffering from rheumatic fever or convalescent from streptococcal disease have been seen to be considerably higher than those observed in studies of healthy populations. Following acute streptococcal pharyngitis in children 6 to 8 years of age, peak antistreptolysin O titers were observed by Streitfeld and Saslaw [9] to range from 250 to 625 Todd units; these titers approximate those observed for adults in the same stage of the disease. Acute rheumatic fever antistreptolysin O serum titer averages have been reported by Rantz [10] to be 405 units; McCarty [11] has found that in this disease titers of 500 units or higher are found frequently.

Comparison of the antistreptolysin O titers of pooled gamma globulin seen in this study with the average serum-antibody levels observed in various healthy and ill population groups indicate the following: (a) The titers of pooled globulin are even higher than the serum titers of patients ill or convalescent from acute streptococcal disease. (b) The antistreptolysin O titers of pooled globulin reflect antibody concentrations 20 to 30 times those observed in sera from healthy adult populations. One commercial pharmaceutical firm* has stated that the gamma globulin concentration of its preparation is approximately 25 times that of normal human plasma. The observed antistreptolysin O titer of its preparation of pooled gamma globulin (1666 Todd units) is approximately 25 times the concentration of antistreptolysin O (71-75 Todd units) found in healthy adult populations under 40 years of age by Streitfeld and Saslaw [7, 8]. This indicates that most if not all of the antistreptolysin O activity of the original pooled-blood samples has been concentrated in the pooled gamma globulin. It is noteworthy that cholesterol titers of pooled gamma globulin as performed in the Pathology Laboratories of the Variety Children's Hospital by the method of Ferro and Ham [12] were negative and thereby indicated either the absence of cholesterol or a concentration below 1 mg%.

Can pooled gamma globulin, by virtue of its antibody content, inhibit the growth of streptococci? Preliminary studies by Streitfeld [13] of the multiplication of Streptococcus pyogenes (Lancefield types 1, 2, 3, 4, and 5) and Group C (human) and G strains of streptococci in Todd-Hewitt broth (Difco) have shown that growth of these organisms, rather than being inhibited, was potentiated by addition to the medium of gamma globulin† in a final concentration of 16.5 mg/ml. These results are reminiscent of the demonstration by Streitfeld et al. [14, 15] that pooled human gamma globulin, despite its high titer against certain of the metabolites implicated in the expression of staphylococcal virulence, promoted rather than retarded the growth in nutrient broth of Staphylococcus pyogenes.

How may gamma globulin, because of its high antistreptolysin O content, function in vivo to modify the toxic effects evoked by infection with S. pyogenes or other streptococci elaborating streptolysin O? Consideration of this problem requires a brief review of the mechanisms whereby the hemolysin has been implicated in the pathogenesis of disease. Partially purified preparations of streptolysin O have been shown by Brittis and Bernheimer [16] to possess toxicity for mammalian lymphocytes as well as for erythrocytes. Ginsburg and Grossowicz [17] have demonstrated in vitro cytotoxic activity of streptolysin O preparations against Ehrlich ascites tumor cells, mouse and rabbit leukocytes, human amnion cells, and chick fibroblasts; antistreptolysin O or cholesterol completely inhibited the cytotoxic action.

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*Pitman Moore Division of Allied Laboratories, Indianapolis, Indiana.

†Poliomyelitis-immune globulin (human) (Lot No. C-583) prepared by Lederle Laboratories Division, American Cyanamid Co. This preparation contained no preservative.

Bernheimer [3] has shown that streptolysin O, when introduced intravenously, is lethal for mice, rats, guinea pigs, rabbits, and cats. Cantoni and Bernheimer [18], and Barnard and Todd [19] observed that, among mice killed by this toxin, intravascular hemorrhage, pulmonary edema, and hemorrhage of the lungs occurred in many of the animals. Post-mortem examinations by Barnard and Todd [19] of mice killed by streptolysin O revealed general venous congestion and pulmonary edema. Reacting streptolysin O with antistreptolysin O or cholesterol prior to injection was shown by Cantoni and Bernheimer [18] to protect animals against the lethal effects of the toxin.

Streptolysin O has been shown to have toxicity for both the amphibian and mammalian heart. Bernheimer and Cantoni [20] induced systolic contracture of the isolated frog's heart by repeated perfusion with solutions containing this exotoxin. Kellner et al. [21] have demonstrated that a minute quantity of streptolysin O could bring about a prompt and irreversible loss of myocardial contractile power when perfused through the isolated, strongly beating hearts of guinea pigs, rabbits, and rats. No decrease in myocardial contractility was obtained when the streptolysin O preparation was not previously activated with cysteine or was inhibited by cholesterol or specific antibody.

Schwab, et al. [22] have reported that through the intravenous injection of reduced streptococcal filtrate containing streptolysin O, the generalized Shwartzman reaction could be provoked in rabbits that had been prepared by the intravenous or intradermal administration of extracts of Streptococcus pyogenes rabbit-skin lesions. Schwab, et al. [23] then found that they could remove the provoking factor from the streptococcal filtrate either by oxidation of the filtrate, or cholesterol, or antistreptolysin O serum. The prominent features of the Shwartzman reaction were death and heart lesions that presented some of the features of myofiber necrosis described by Thomas, et al. [24] and resembled in some respects the cardiac lesions reported by Murphy and Swift [25] in rabbits subjected to repeated streptococcal infections. Thomas [26] has emphasized the importance of these observations by his concept that the generalized Shwartzman reaction in rabbits may serve as an experimental model for rheumatic fever.

The possible role of antistreptolysin in the attenuation of clinical streptococcal infection remains to be determined. Hamburger and Lemon [27] have demonstrated that patients with high serum titers of antistreptolysin O were not protected against the development of new Streptococcus pyogenes infections. However, resistance to infection has been associated by Kuttner and Lenert [28], Rantz, et al. [29], and Lancefield [30] with the presence of M type-specific antibodies that function in vivo or in the presence of whole blood; Lancefield [31] has indicated that phagocytosis and leukocytic bactericidal activity are promoted by these antibodies. Consideration of the implications of the present demonstration of high antistreptolysin O titers in gamma globulin preparations and the reports by Fisher [1] and Fisher and Manning [2] of the therapeutic activity of gamma globulin in experimental Streptococcus pyogenes infections have now lead to the postulation that gamma globulin may contain a high content of antibodies other than antistreptolysin O that are potent in the prophylaxis or attenuation of streptococcal infection. Experimental studies of the effectiveness of gamma globulin against the other streptococcal components and metabolites implicated in the in vivo expression of virulence currently are in progress.

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. James Jablon of the National Children's Cardiac Hospital, Miami, for the study strains of Streptococcus pyogenes, and to Dr. Michael Bevilaqua and Mrs. Carol Cox of the Pathology Laboratories of the Variety Children's Hospital, Miami, for the performance of cholesterol assays on preparations of pooled gamma globulin.

The preservative-free pooled gamma globulin used in this study was generously supplied by the Lederle Laboratories Division, American Cyanamid Co.

SUMMARY

Pooled normal human gamma globulin has been seen to exert therapeutic activity and to markedly potentiate antibiotic activity in Streptococcus pyogenes infections. In seeking to understand the mechanism of this protection it was decided to investigate the effect of gamma globulin on the various streptococcal components and metabolites implicated in virulence. Here reported are the results of the pilot study, the in vitro titration of gamma globulin activity against the exotoxin, streptolysin O. It was found that: (a) Streptolysin O hemolytic activity was inhibited by very low concentrations of gamma globulin; antistreptolysin O titers were 20 to 30 times the average serum titers previously observed in surveys of normal adult human populations; (b) Seven different commercial preparations of pooled human gamma globulin were equally effective as inhibitors of the hemolytic activity of streptolysin O. The roles of streptolysin O and its antibody inhibitor in the pathogenesis of streptococcal disease were discussed. The significant effect of gamma globulin on streptolysin O may account in part for its demonstrated therapeutic efficacy. It is suggested that pooled human gamma globulin may contain other antibodies capable of modifying streptococcal infection.

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DISCUSSION

DR. BONDI: Have you had an opportunity yet to test the gamma globulin for antihyaluronidase?

DR. STREITFELD: No. These titrations are actually in progress right now. I might indicate that in previous studies we tested the gamma globulin activity against staphylococcal coagulases, both bound and free, and we found that it had tremendously high titers against these staphylococcal virulence factors.

THE USE OF BLOOD SERUM AS TEST MEDIUM IN THE IN VITRO EVALUATION OF ANTIBIOTICS

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INTRODUCTION

A considerable amount of information used in the evaluation of new antibiotics is derived from in vitro studies. Until clinical investigations are complete impressions as to therapeutic effectiveness are obtained from in vitro bacteriostatic and bactericidal tests employing prepared bacteriological media. Since we are concerned about the concentration of antibiotics obtainable in blood, the possible binding effect of serum, and ultimately the action of the drugs in the human body, it occurred to us that the action of antibiotics on microorganisms in serum should be given some attention. In these studies blood serum was used as a test medium on the premise that it would be more representative of body fluids than are most bacteriological media.

PRELIMINARY OBSERVATIONS

In some early experiments to determine the bactericidal action of various antibiotics versus cultures of Staphylococcus aureus in normal human serum we observed that S. aureus strains grew in serum without the addition of nutrients. An investigation of the reason for some unpredicted results also revealed that the pH of the serum changed during incubation from the normal range of 7.2-7.4 to 8.4-8.6. The rise in pH was apparently due to loss of CO_2 from the natural bicarbonate buffering system of the serum since bubbling CO_2 into the serum lowered the pH. These findings were in agreement with those of Ekstedt and Nungester [1], who reported that coagulase-positive strains of S. aureus grow in human serum and that capping the tubes of serum with rubber stoppers prevented pH shift due to CO_2 loss. Since variations in the pH of testing media greatly affect the activity of some antibiotics [2, 3] it is necessary to standardize the pH of sera used in testing. Sera were adjusted to pH 7.0-7.1 just prior to use as test media by bubbling CO_2 into them. This was accomplished by the use of a simple apparatus. Crushed dry ice was put into a rubber-stoppered 250-ml side-arm vacuum flask. A sterile cotton-plugged pipette was attached to the side-arm of the flask with a length of rubber tubing. The gas issuing from the pipette was bubbled into chilled serum, which was being checked simultaneously on a pH meter whose electrodes had been cleaned with ethanol. After this pH adjustment the container was capped and the serum kept cold while being handled since CO_2 is more rapidly lost at higher temperatures. Tubes of serum were plugged with rubber stoppers. This prevented CO_2 loss and allowed a pH rise of only 0.2-0.3 of a unit during incubation. We observed that when CO_2 is bubbled into chilled serum the pH drops slowly to about 6, at which point the buffering capacity is apparently exhausted and the pH drops rapidly. When the stream of CO_2 is removed from the serum the pH rises rapidly to about 6, at which point the buffering system again becomes effective and the pH rises slowly as CO_2 is lost.

EXPERIMENTAL

Normal human serum and normal horse serum were used in these studies. The sera were removed aseptically from samples of clotted whole blood and were stored, frozen, or chilled in screw-capped or rubber-stoppered bottles. Small tubes (13 × 100 mm) fitted with white 00 soft rubber stoppers were employed in all tests.

Several microbial species were checked to determine if they would grow in serum without nutrients added. Those tested were *Staphylococcus aureus* 209P, *Staphylococcus albus* X21, *Bacillus subtilis* ATCC 6633, *Sarcina lutea* ATCC 10054, *Mycobacterium* sp. 607, *Mycobacterium avium* ATCC 7992, *Escherichia coli* ATCC 4157, *Proteus vulgaris* ATCC 9484, *Pseudomonas aeruginosa* ATCC 9027, *Aerobacter aerogenes* ATCC 8308, *Klebsiella pneumoniae* X83, *Salmonella enteritidis* ATCC 9221, *Shigella paradysenteriae* X87, *Brucella bronchiseptica* NRRL B-140, *Vibrio metschnikovii* X253, *Saccharomyces pastorianus* X52, and *Candida albicans* A17. Suspensions of about 0.10 optical density were prepared in sterile distilled water from slants of each culture and 0.05-ml portions of each suspension were pipetted into four replicate tubes. One-milliliter aliquots of chilled human or horse serum previously adjusted with CO_2 as described above were pipetted into duplicate tubes of each organism. These were stoppered immediately, shaken, incubated at 37 C, and observed for growth after incubation for 20 hr and 2 days. Uninoculated serum controls were similarly prepared to check sterility and pH after incubation. The growth of the test organisms in the two kinds of serum is recorded in Table 1.

Table 1. Growth of Microorganisms in Serum Without Added Nutrients

Test organism	Horse serum		Human serum	
	20 hr	2 days	20 hr	2 days
Staphylococcus aureus	+++	+++	+++	+++
Staphylococcus albus	+++	+++	+++	+++
Bacillus subtilis	–	–	–	–
Sarcina lutea	–	++	–	++
Mycobacterium sp.	–	–	–	–
Mycobacterium avium	–	++	–	++
Escherichia coli	+	+++	–	–
Proteus vulgaris	++	+++	–	–
Pseudomonas aeruginosa	–	+++	–	–
Aerobacter aerogenes	–	+++	–	++
Klebsiella pneumoniae	–	+++	–	–
Salmonella enteritidis	–	+++	–	–
Shigella paradysenteriae	–	++	–	–
Brucella bronchiseptica	–	++	–	–
Vibrio metschnikovii	–	–	–	–
Saccharomyces pastorianus	–	–	–	–
Candida albicans	++	++	+++	+++
Controls (uninoculated)	–	–	–	–

Key: +++ good growth, ++ fair growth, + very poor growth, – no growth.

Of the cultures tested the staphylococci grew best in both kinds of serum. They formed a thick layer of cells at the bottom of the tubes and the serum appeared quite turbid when shaken. The viable count at 20 hr in serum was approximately $1 \cdot 10^8$ cells/ml or about one-tenth the number which grows in a rich laboratory medium such as brain-heart infusion (Difco) broth.

Because of the interest in the activity of antibiotics against staphylococci the minimum bacteriostatic concentrations of chloramphenicol, chlortetracycline, desmethyltetracycline, oxytetracycline, tetracycline, erythromycin, erythromycin propionate, desmycosin, tylosin, kanamycin, neomycin, novobiocin, oleandomycin, triacetyloleandomycin, penicillin G, penicillin V, α-phenoxyethylpenicillin, ristocetin, streptomycin, and vancomycin versus *S. aureus* 209P were determined in pooled human serum, horse serum, and brain-heart infusion (Difco) broth. Serial twofold dilutions were made in sterile distilled water from 1-mg/ml solutions of each antibiotic. One-tenth-milliliter aliquots from the stock solutions and from each dilution thereof were transferred into three replicate tubes so that for each antibiotic three series of identical concentrations were prepared. These were pipetted in carefully at the bottom of the tubes. Aliquots of brain-heart infusion (Difco) broth, chilled pH-adjusted human serum, and chilled pH-adjusted horse serum were each inoculated with 0.1% of an 18-hr culture of *S. aureus* 209P grown in brain-heart infusion (Difco) broth; 0.9-ml portions of these were pipetted into the three series of tubes so that identical concentrations of the antibiotics were contained in 90% broth, 90% human serum, and 90% horse serum. The serum tubes were stoppered immediately after filling to prevent loss of CO_2 and all tubes were shaken to mix the antibiotic with the inoculated serum or broth. The tubes were incubated 20 hr at 37 C and the minimum concentrations producing complete inhibition of the test organisms, judged by visual observation, were recorded. The results are shown in Table 2.

Table 2. The Minimum Concentrations (μg/ml) of Antibiotics Inhibitory to *Staphylococcus aureus* 209P in 90% Human Serum, 90% Horse Serum, and Brain-Heart Infusion (Difco) Broth

Antibiotic	Human serum	Horse serum	BHI broth
Neomycin	3.13	6.25	12.5-25.0
Streptomycin	3.13	6.25	50-100
Kanamycin	12.5	6.25	50
Tylosin	0.39	0.78	0.78-1.56
Desmycosin	0.78	0.78	1.56
Ristocetin	6.25	6.25	12.5
Vancomycin	1.56	1.56	1.56
Triacetyloleandomycin	6.25	6.25	6.25
Oleandomycin	1.56	3.13	1.56
Penicillin G	0.025	0.05	0.025
Penicillin V	0.05	0.05	0.025
α-Phenoxyethylpenicillin	0.1	0.1	0.05
Erythromycin propionate	1.56	0.78	0.78
Erythromycin	1.56	0.78	0.39-0.78
Tetracycline	3.13	3.13	0.39-0.78
Chlortetracycline	6.25	3.13	0.78
Oxytetracycline	3.13	3.13	0.78
Desmethyltetracycline	3.13	3.13	0.39
Chloramphenicol	25.0	25.0	6.25
Novobiocin	100.0	12.5	0.2-0.39

It is interesting to compare the minimum concentrations of antibiotics inhibitory in broth and serum. Some inhibited at the same level both in serum and in broth. Some were more potent in serum than in broth and others were less potent in serum than in broth. The decrease in activity of some antibiotics in serum as compared to broth is probably due in part to protein binding; the reason some antibiotics are more potent in serum than in broth remains to be determined.

In a similar manner several cultures of S. aureus were tested in pooled human serum and brain-heart infusion (Difco) broth versus penicillin G, penicillin V, and α-phenoxyethylpenicillin. The cultures were S. aureus Heatley, S. aureus 3055, S. aureus 209P, S. aureus 209P (PR), S. aureus H431, S. aureus H450, S. aureus H461, and S. aureus H470. S. aureus 209P (PR) is a strain with laboratory-induced resistance to penicillin G that tolerates up to 5 mg/ml of that antibiotic in broth and does not destroy it. Cultures bearing the designation "H" were clinical penicillin-resistant strains which destroyed penicillin and were selected for this test because they had exhibited varying degrees of resistance to penicillin G. Solutions of the penicillins (1 mg/ml) were diluted in sterile distilled water to obtain serial twofold concentrations and 0.1-ml portions of each were carefully pipetted into the necessary number of replicate tubes to which was then added 0.9 ml of inoculated serum or inoculated broth. The inocula were 0.1% of overnight cultures of the test organisms grown in brain-heart infusion (Difco) broth. The chilled serum, prior to being inoculated, was adjusted with CO_2 to pH 7. The tubes to which inoculated serum was added were immediately stoppered and all tubes were agitated to mix the penicillin with the seeded broth or serum. After 20 hr incubation the minimum inhibitory concentrations were recorded as is shown in Table 3.

Table 3. Minimum Inhibitory Concentrations (μg/ml) of Penicillins vs Staphylococcus aureus Cultures in 90% Broth and 90% Human Serum

Test organism	90% broth			90% human serum		
	I	II	III	I	II	III
S. aureus (Heatley)	0.025	0.025	0.05	0.025	0.05	0.1
S. aureus 3055	0.025	0.025	0.05	0.05	0.1	0.2
S. aureus 209P	0.025	0.025	0.05	0.025	0.05	0.1
S. aureus 209P (PR)	>100	>100	>100	0.2	0.39	0.78
S. aureus H431	12.5	6.25	0.78	12.5	6.25	3.13
S. aureus H450	6.25	3.13	0.78	3.13	1.56	1.56
S. aureus H461	>100	>100	>100	100	100	25.0
S. aureus H470	50	50	1.56	25	12.5	6.25

I = Penicillin G
II = Penicillin V
III = α-Phenoxyethylpenicillin

The differences between the concentrations of a penicillin-inhibiting sensitive strain of staphylococci in broth and in serum are probably due to serum binding. The concentration of a penicillin necessary to inhibit a clinical resistant culture may depend upon the ability of the organism to produce penicillinase in the test medium, as well as upon the activity of penicillinase versus penicillin in the test medium. It may be interesting to note that the culture with very high laboratory-induced penicillin-resistance in broth is so completely sensitive in serum. For this reason studies concerning the resistance of staphylococci might be more meaningful if tested for sensitivity in pooled human serum as the test medium.

ACKNOWLEDGMENT

The technical assistance of Miss Stanis Stroy and Mr. James Solenberg is gratefully acknowledged.

SUMMARY

Blood serum was studied as an antimicrobial test medium because it is more closely related to body fluids than are prepared bacteriological media. Staphylococcus aureus cultures grow very well in serum, making this important species available for testing in serum. Use of serum as a bacteriological testing medium should supply useful information concerning serum binding, antibiotic resistance, and bactericidal action, and might add significance in other ways to in vitro studies of antimicrobial agents for human use.

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DISCUSSION

DR. BONDI: Is it true that the antibiotics showing the greatest binding or those where serum had the greatest effect in decreasing activity were essentially what we earlier in the afternoon called "static" agents, as compared with the "cidal" agents, which seemed to be at the top?

DR. HOEHN: That is correct. Chloramphenicol, the tetracyclines, and novobiocin all came at the bottom of the group. Yet they were the most active antibiotics in broth.

Q: I'm not certain that I completely saw the first chart shown, but in your list of organisms growing in serum I don't recall if you used the Streptococcus or not. I might indicate that this grows extremely well, which makes another organism available for this type of testing.

DR. HOEHN: We just had the two strains of Staphylococcus aureus and Staphylococcus albus in the chart. The purpose of this paper was to present the information in the hope that it might be added as a tool in the over-all antibiotic picture.

COMPARATIVE STUDIES OF FIVE ANTIBIOTICS HAVING ANTIBACTERIAL SPECTRA SIMILAR TO THAT OF ERYTHROMYCIN

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INTRODUCTION

In an earlier study [1] comparing the in vitro and in vivo activity of erythromycin and spiramycin we found that spiramycin was much less effective than erythromycin in vitro, but in in vivo tests with experimental animals (mice) it displayed a much greater affinity for tissue. Spiramycin was retained in the tissues for more than 24 hr following a single subcutaneous injection, and relatively high concentrations were attained in organs during prolonged treatment. Erythromycin, on the other hand, was eliminated from the body within 15-18 hr after its administration. Similar findings were reported by Benazet and Dubost [2] and Pellerat et al. [3]. The present paper is a report on the continuation and extension of these earlier studies with erythromycin and spiramycin to include carbomycin, oleandomycin, and novobiocin with particular emphasis on the affinity of these drugs for different organs of the mouse.

PROCEDURE AND RESULTS

In vitro tests. One milliliter of varying concentrations of the antibiotics was added to tubes containing 9.0 ml of Todd-Hewitt medium [4] to give final concentrations ranging from 0.0125 to 1.6 μg/ml. The tubes were inoculated with one drop of an 18-hr broth culture of Diplococcus pneumoniae T_2 adjusted by photometer readings to contain $2 \cdot 10^6$ organisms/ml and were incubated at 37 C for 24 hr. Growth was then stopped by adding 0.5 ml of formaldehyde to each tube. The end point was determined by visual examination. The results are shown in Table 1.

Erythromycin was found to have the greatest antibacterial activity, being four times as effective as spiramycin and carbomycin, and 16 and 32 times more effective than oleandomycin and novobiocin, respectively.

Table 1. The Inhibitory Concentrations of Antibiotics Tested Against Diplococcus pneumoniae T_2

Antibiotic	Minimal inhibitory concentration, μg/ml
Erythromycin	0.025
Spiramycin	0.1
Carbomycin	0.1
Oleandomycin	0.4
Novobiocin	0.8

Table 2. Concentration of Antibiotic in the Organs of Mice Following Subcutaneous Injection of 100 mg/kg

Time, hr	Organ	Concentration of antibiotics, μg/g of organ									
		Spiramycin		Erythromycin		Oleandomycin		Novobiocin		Carbomycin	
		a*	b	a	b	a	b	a	b	a	b
3	Lungs	62.5	114	21	17.5	39	20	11.8	39	10	8
	Spleen	52.5	77.5	16.5	14	32	11	0	18	8.5	8
	Kidney	42	41.5	20	16	48	12	12	22	8	4
	Liver	21	30	40	18.5	50	8	34	48	2	0
6	Lungs	41	62.5	5	2.5	6.5	Trace	0	0	2	1.2
	Spleen	35	62	4.25	1.7	8	0	0	0	2	Trace
	Kidney	33	41.5	4.5	2.5	4	Trace	0	0	3	0
	Liver	32.5	24	5.5	2.8	Trace	0	10	18	Trace	0
15-18	Lungs	17.3	32.5	0.5	0	0	0	0	0	0	0
	Spleen	15	49	0.6	0	0	0	0	0	0	0
	Kidney	10.3	26.5	0.6	0.5	0	0	0	0	0	0
	Liver	4.5	6	0.5	0	0	0	0	0	0	0
24	Lungs	11.5	14	All others gave negative results.							
	Spleen	11.5	24								
	Kidney	10	23								
	Liver	1.8	0								

*a and b represent separate experiments. Each figure represents the average concentration in the organs of two mice.

In vivo tests. To determine the distribution of antibiotic in body organs, albino mice* (five groups, 16 mice/group) weighing 25–28 g were injected subcutaneously with 100 mg/kg of antibiotic. Each group received a different antibiotic. Two mice from each group were sacrificed at 3, 6, 15-18, and 24 hr after administration of the antibiotic and their lungs, spleen, kidneys, and livers were removed and ground separately in pyrex tissue grinders. The tissues from each organ were suspended individually in the proportion of 1 g of tissue to 10 ml of phosphate buffer (pH 7.0 for spiramycin and pH 8.0 for all the others), shaken gently for 10 min on a wrist-action shaker, and then centrifuged at 2000 rpm for 15 min. The supernate was withdrawn and assayed for antibiotic content by plate methods similar to those described by Grove and Randall [5]. The experiment was carried out twice and the results are shown in Table 2. The highest concentration of antibiotic in the organs was found with spiramycin; not only was its concentration much higher than than of the others after 3 hr but it was retained in the tissues at high levels for a much longer period—over 24 hr. The next highest concentration was found with erythromycin, which showed high levels for at least 6 hr but dropped markedly after 15 and 24 hr. Of the remaining three antibiotics, the highest initial concentration was observed with oleandomycin, followed by novobiocin, with carbomycin showing the lowest concentration. Oleandomycin showed reasonably good concentrations after 3 hr but the levels of all of the latter three antibiotics dropped rapidly and only traces were detectable after 6 hr and none was detectable after 15 hr.

Table 3. Protective Action of Antibiotics Against Pneumococci in Mice*

Antibiotic	Time of challenge following antibiotic injection, hr	Number of mice	Number surviving						
			1 day	2 days	3 days	4 days	5 days	6 days	7 days
Spiramycin	0	20	20	20	20	20	20	20	20
	3	20	20	20	20	20	20	20	20
	6	20	20	20	20	20	20	20	20
Erythromycin	0	20	20	20	8	8	8	8	8
	3	20	20	20	4	4	4	4	4
	6	20	20	20	0	0	0	0	0
Oleandomycin	0	20	17	0	No survivors				
	3	20	14	0					
	6	20	4	0					
Carbomycin	0	20	15	0					
	3	20	9	0					
	6	20	1	0					
Novobiocin	0	20	8	0					
	3	20	6	0					
	6	20	0	0					

*Antibiotic was injected subcutaneously 100 mg/kg. The challenge dose was 80,000 Diplococcus pneumoniae T_2 intraperitoneally. Twenty mice from the control group were injected at each challenge period and all died within 24 hours.

Mouse-protection studies were performed to determine the protective action of antibiotics remaining in the tissue. Albino mice (five groups, 60 mice/group) weighing 25-28 g were injected subcutaneously with 100 mg/kg of antibiotic. A separate group of 60 mice was used as a control. Immediately following injection of the antibiotic (one operator administered the antibiotic and another the challenge dose) and at intervals of 3 and 6 hr afterward 20 mice from each group, including the control group, were challenged intraperitoneally with 4 MLD of D. pneumoniae T_2 (approximately 80,000 organisms) and observed for a period of seven days. The results are shown in Table 3. All of the mice treated with spiramycin survived the challenge dose. Of the mice receiving erythromycin, 8 (40%), 4 (20%), and none survived the immediate, 3-hr, and 6-hr challenge respectively. None of the mice receiving oleandomycin, novobiocin, or carbomycin survived any of the challenge doses.

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*Obtained from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario.

DISCUSSION

In evaluating an antibiotic one should consider its in vitro activity, its toxicity, its chemical behavior or stability in the body, and its absorption, retention, and elimination by the body. The first two may be established by straightforward and routine testing procedures but what happens to the antibiotic in the body is much more difficult to assess. Until recently it was considered that serum-level determinations gave most of the essential answers. A high and constant blood level was considered indicative of good absorption and slow excretion. This concept, however, has been questioned seriously by a number of workers. Dost and Weingartner [6] have pointed out that blood levels alone may not be a true gauge of the effectiveness of an antibiotic since there need be no direct relationship between the concentration of antibiotic in the blood and that in the tissues, and further, that the concentration in the tissues is most important when the infection is deep seated in the tissue. Similar points of view were expressed by Sassinot and Chabbert [7].

Novobiocin presents a good case in point. Taylor et al. [8], Martin et al. [9], and Wright et al. [10], found that high blood levels followed the administration of novobiocin and they considered this an advantage. However, in distribution experiments Spitzy and Hitzenberger [11] noted that novobiocin, unlike a number of antibiotics such as erythromycin and oleandomycin, was confined almost entirely to the blood stream, and this was a definite disadvantage.

In the present studies using D. pneumoniae T_2 as the test organism, erythromycin was found to be the most effective antibiotic in vitro. It was four times as effective as spiramycin and carbomycin and 16 and 32 times more effective than oleandomycin and novobiocin, respectively.

In studying the pharmacodynamic activity of these drugs, however, it was found that only spiramycin, erythromycin, and to a somewhat-lesser extent, oleandomycin exhibited affinity for tissues. The results in our earlier studies were confirmed in that spiramycin again exhibited a much more marked tissue affinity than erythromycin. The remaining antibiotics exhibited little affinity for tissues and only traces were detectable in the organs 6 hr after administration (Table 2).

In the animal-protection experiments described here it was found that the antibiotics exhibiting affinity for tissue were much more capable of protecting animals against subsequent infection than those not showing affinity. Spiramycin was found to have the greatest affinity for tissue and all animals treated with this antibiotic survived the challenge dose. However, erythromycin, which exhibited only a slightly greater affinity for tissues than oleandomycin but which was 16 times more active in vitro against the test organism, gave much better protection than oleandomycin. Therefore, both the in vitro activity and the tissue affinity must be taken into consideration in the evaluation of an antibiotic.

We have recently completed a series of preliminary experiments in monkeys. When the five antibiotics being considered in this study were injected subcutaneously the pattern of tissue affinity was identical to that found with mice. This would suggest that the results of the animal experiments might well apply to man. In determining the fate of an antibiotic in man, however, one must consider its route of administration. One could not, for example, always expect that the results obtained following the parenteral administration of an antibiotic to experimental animals will necessarily be similar to those obtained following its oral administration to man. In the latter case the rate of absorption through the alimentary tract would influence the results greatly. Lepper et al. [12] and Jones et al. [13] have both shown that spiramycin is absorbed poorly through the human gut. This has been confirmed by our own preliminary experiments with humans (results not shown here) in which nine adults were dosed with capsules of spiramycin base (the commercial product for adult use) prior to lobectomy and the lungs excised either 5 or 17 hr after treatment with the antibiotic. The lung tissue of only one was found to contain spiramycin. However, in similar experiments using spiramycin adipate (the commercial product for pediatric use), all of six other test persons absorbed the drug, and relatively high quantities were found in the lung tissues. It would, therefore, seem clear that therapeutic failures with spiramycin, in some instances, could be attributable to the poor absorption qualities of spiramycin base.

The route of administration, the rate of absorption, the stability of the antibiotic, and its distribution and retention by tissues are of primary importance for, as has been pointed out elsewhere [14], it matters very little if an organism is sensitive to an antibiotic if that antibiotic cannot reach the source of infection in sufficient quantity to be effective. It would seem clear that blood-level determinations alone do not tell the whole story. Drug manufacturers would provide a very useful service and the claims made for their products would be more solidly based if they provided such data.

ACKNOWLEDGMENTS

The collaboration of Dr. D. G. Gray of the Royal Ottawa Sanatorium is gratefully acknowledged.

SUMMARY

Comparative in vitro and in vivo tests were conducted on five antibiotics having similar antibacterial spectra. Using the tube-dilution test and Diplococcus pneumoniae T_2 as the test organism the effectiveness of these drugs in vitro fell in the following order: erythromycin, spiramycin, carbomycin, oleandomycin, and novobiocin.

In studying the pharmacodynamic activity of these drugs it was found that only spiramycin, erythromycin, and to a lesser extent oleandomycin exhibited affinity for body tissue. In animal-protection experiments it was found that the antibiotics exhibiting the greatest affinity for tissue were more capable of protecting animals against subsequent infections.

The significance of these findings is discussed and consideration is given to the factors concerned in the evaluation of an antibiotic.

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DISCUSSION

DR. FLYNN (Eli Lilly Company): Dr. Greenberg, I wonder why you pick a pH of 7 to extract spiramycin whereas you picked pH 8 for your buffer for your extraction of all the other antibiotics.

DR. GREENBERG: The fact is that when we first started working with spiramycin we didn't have a great deal of experience with it and we started with a pH 7 buffer. For that reason we kept right on going. However, we have done some work with pH 8 buffer and there was no difference between the pH 7 and the 8.

DR. FLYNN: Did you find this to be true of the other antibiotics also?

DR. GREENBERG: The erythromycin seems to do better with pH 8 than with pH 7.

LABORATORY OBSERVATIONS ON THREE ERYTHROMYCIN DERIVATIVES IN A TRIPLE CROSSOVER STUDY

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INTRODUCTION

Erythromycin, like other antibiotics such as penicillin and the tetracyclines, has been repeatedly modified after successful clinical and laboratory evaluations in relation to infectious processes, in efforts to find a more efficient form of the drug. Recently two such modifications became available for comparative studies, erythromycin propionate* in a 250-mg gelatin capsule and erythromycin stearate in the form of an enteric coated tablet whose contents were assayed to be equivalent to 250 mg of erythromycin base.

The clinical and laboratory evaluation of erythromycin base has shown that this antibiotic is most effective in infections caused by Gram-positive bacteria such as staphylococci, streptococci, or enterococci [1–4]. The limited number of sensitivity studies done in this and other laboratories would indicate that generally the susceptibilities of Gram-positive organisms in vitro are similar for all three of the erythromycin preparations.

Salitsky [3] reported that erythromycin propionate in an absorption crossover study in ten healthy males produced higher and earlier rises of antibiotic activity in serum than equivalent amounts of erythromycin base. Griffith [5], Hirsh [6], and Clapper [7] reported that the propionate form produced greater activity in blood sera than did equivalent dosages of erythromycin stearate in small groups of patients. These observations were further substantiated when Griffith [5] and Blough [8] undertook comparative absorption determinations of the two erythromycin derivatives. However, it has also been reported that the activity in sera of patients receiving either erythromycin propionate or erythromycin stearate was markedly depressed by preliminary ingestion of food [5, 7, 9]. Sylvester and Josselyn [10], on the other hand, using an oral suspension of erythromycin stearate concluded from their limited study that the intake of food prior to administration of the medication had little or no effect on the activity assayable in the blood.

This study was undertaken to compare the efficiency in absorption and rate of excretion following oral administration of the three erythromycin preparations utilizing the triple crossover technique. Antibiotic activity in blood sera and urine was determined in 60 carefully selected hospital patients. It was our opinion that this large group of subjects tested under the triple crossover method would provide conclusive data on the erythromycin activity in body fluids in relation to time factors.

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*The trade names of Eli Lilly and Company for erythromycin base and erythromycin propionate are Ilotycin and Ilosone, respectively. Erythromycin stearate is marketed by Abbott Laboratories under the name Erythrocin Stearate.

METHODS

Preselected patients in this study received no chemotherapeutic agents which might interfere with the interpretation of the results for at least one week prior to, or at any time during, the investigation. Any patient whose control body-fluid specimens exhibited antibacterial activity was automatically disqualified from participation. No special consideration as to diet or treatment was accorded the subjects other than that received in routine service and available to ward patients throughout the hospital.

The 60 patients were divided into two groups based on the quantity of the erythromycin derivatives they were to receive. The first group of 38 patients received a single 250-mg oral dose of any one of the three erythromycin preparations, and then blood and urine samples were collected and assayed. An interval of one week was allowed to elapse to insure a negative control specimen prior to repeating the process with the next form of erythromycin. This process was repeated until all three medications had been taken by each subject. The above procedure was repeated with the second group of 22 patients, except that they received a 500-mg oral dose. After completion of this phase of the study, ten patients were chosen from each group for additional assays to determine the effect of food on the serum-antibiotic levels.

Throughout the study, premedication specimens were used as controls. Blood specimens were also collected at 1, 2, 3, 6, and 8 hr after medication and centrifuged to separate the serum from the cells. The serum was drawn off aseptically, stored at −15 C, and assayed the following day. Urine specimens were obtained as controls and at 4, 8, 12, and 24 hr after medication. The total volume collected at a designated interval was recorded, a 10-ml sample was centrifuged, and the supernatant stored in a deep freeze until processed. Both urine and blood specimens from patients were assayed simultaneously.

A modified cup-plate method [11, 12] using Sarcina lutea PCI 1001 was employed for the antibiotic activity determinations. For the assay of each erythromycin preparation a standard curve was drawn using an assayed dry sample of the respective antibiotic. This curve, with appropriate correction factors, was

Table 1. Average Serum Concentrations, μg/ml, Following Administration of Single Oral Doses of Three Erythromycin Preparations to Fasting Patients

Specimen, hr	Dosage, mg	Number of patients	Erythromycin base	Erythromycin propionate	Erythromycin stearate
Control	250	38	NI*	NI	NI
1	250	38	0.24	0.11	0.11
2	250	38	1.27	1.6	0.9
3	250	38	0.82	1.76	1.06
6	250	38	0.87	0.58	0.6
8	250	38	0.11	0.33	0.25
Control	500	22	NI	NI	NI
1	500	22	0.58	0.45	0.14
2	500	22	1.25	1.43	1.43
3	500	22	1.0	1.95	1.47
6	500	22	0.85	1.55	1.0
8	500	22	0.1	1.29	0.9

*No inhibition.

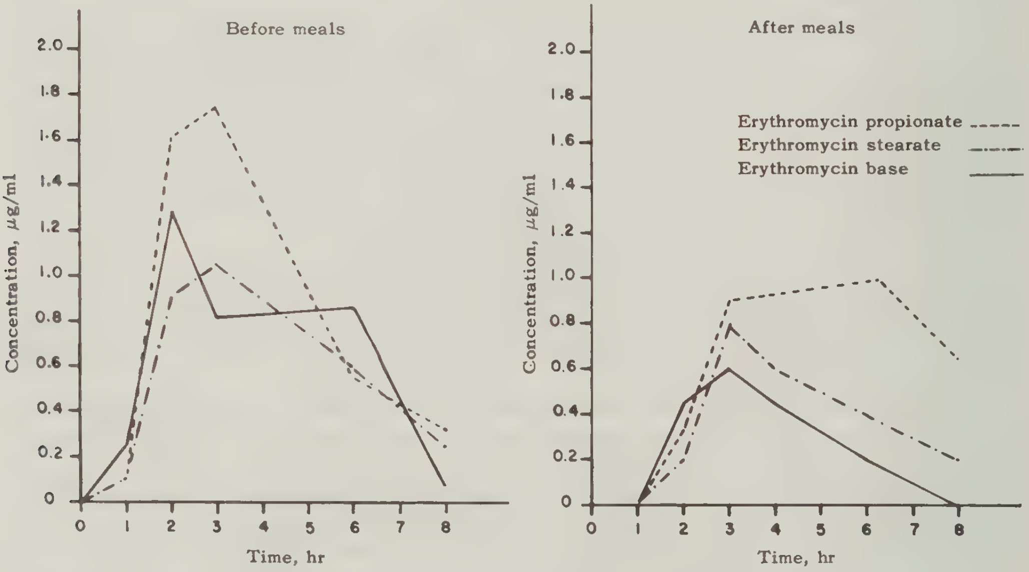
used to estimate the concentration of each form of medication in blood and urine samples.

RESULTS

A tabulation of the average concentration of the erythromycin activity in sera at various intervals after patients received a single oral dose of 250 mg or 500 mg before meals is recorded in Table 1 for each of the medications and is also presented graphically in Figs. 1 and 3.

It is apparent that in patients who received 250 mg of erythromycin base before meals the peak concentration of activity was found after 2 hr while for the propionate and stearate forms it appeared after 3 hr. During the first hour after medication the base was absorbed faster than the two derivatives. However, by the second hour the rates of absorption of erythromycin propionate and stearate were relatively as great as that of the base, if not greater, as indicated by Fig. 1.

At the second hour the average activity of the propionate derivative (1.6 μg/ml) exceeded that of the base or the stearate form and the concentration of the base (1.27 μg/ml) was greater than that of the stearate (0.9 μg/ml).



Figs. 1 and 2. Average erythromycin activity in sera, μg/ml, following 250-mg oral dose before and after meals.

The 3-hr specimens exhibited activity for the propionate which had increased to 1.76 μg/ml and to 1.06 μg/ml for the stearate while the levels for the base began to diminish (0.82 μg/ml).

After the third hour the activity for all three preparations diminished steadily as indicated by the 6- and 8-hr samples. Eight hours after initiation of treatment the average levels for erythromycins propionate and stearate (0.33 and 0.25 μg/ml respectively) were greater than for the base (0.11 μg/ml).

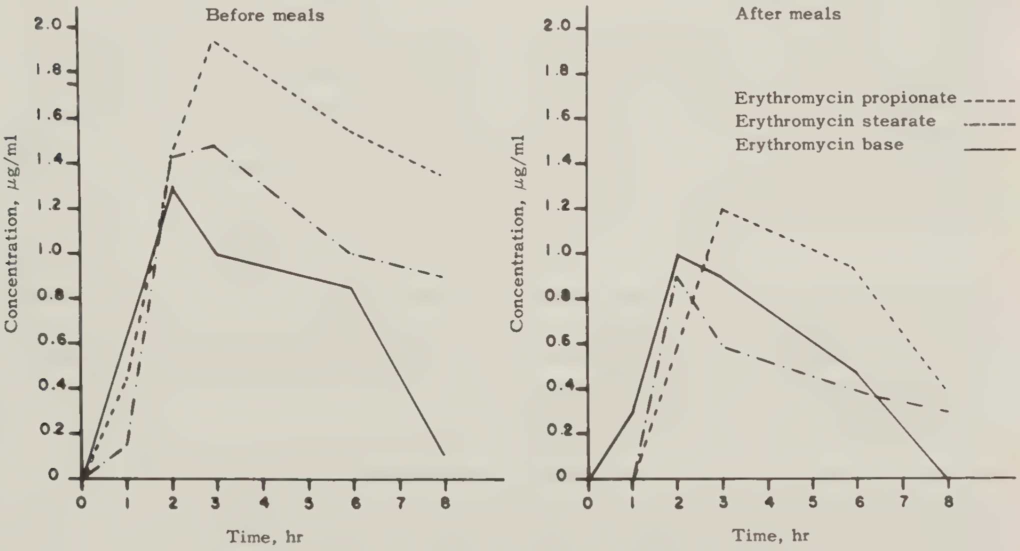
It is also interesting that the average percent excretion of the total dose of the antibiotics was greater for the base at the end of the 24-hr period (8.25%) as compared to that of the propionate (5.57%) and the stearate (5.5%).

Table 2. Average Serum Concentrations, μg/ml, Following Single Oral Doses of Three Erythromycin Preparations Given After Meals

Specimen, hr	Erythromycin base	Erythromycin propionate	Erythromycin stearate
Control	NI*	NI	NI
1	NI	NI	NI
2	0.45	0.3	0.2
3	0.6	0.9	0.8
6	0.2	1.05	0.4
8	NI	0.7	0.2
Control	NI	NI	NI
1	0.3	NI	NI
2	1.05	0.6	0.9
3	0.9	1.2	0.9
6	0.48	1.0	0.6
8	NI	0.4	0.3

*No inhibition.

The results with the second group of 22 patients, who received a single 500-mg oral dose before breakfast (Fig. 3), showed that the highest or peak concentration of erythromycin activity in blood sera was achieved with the propionate derivative at 3 hr posttreatment (1.95 μg/ml). The next highest, 1.43 μg/ml at 2 hr and 1.47 μg/ml at 3 hr, was achieved with the stearate while for the erythromycin base the greatest degree of activity, 1.25 μg/ml, occurred 2 hr posttreatment. At 8 hr after administration of medication the average blood level for the propionate was 1.29 μg/ml and for the stearate, 0.9 μg/ml, which is nine times or more greater than that of the base. The average concentration in blood after erythromycin propionate was one and one-half times greater than that of the



Figs. 3 and 4. Average erythromycin activity in sera, μg/ml, following 500-mg oral dose before and after meals.

stearate derivative. Here again, with the doubled quantity of the three medications, a greater amount of the base (10.59%) was excreted at the end of 24 hr as compared with the propionate (6.79%) and the stearate (5.46%). In this group of patients all three forms of erythromycin produced greater peak concentrations in the blood and also greater concentrations 8 hr after treatment.

In Table 2 and Figs. 2 and 4 we summarize the results in terms of the average concentration of the antibiotics in blood sera when they were administered after breakfast. It is evident that the consumption of food before ingestion of any of the three antibiotics depressed the activity assayable in blood sera.

DISCUSSION

Previous observations have shown that the sensitivity of Gram-positive organisms to erythromycin base and to the propionate and stearate derivatives is generally similar. Therefore, the antibiotic with the capacity to induce the greatest antibacterial activity for the longest period of time on equivalent dosage may be considered the most efficient. It is evident from our results that erythromycin propionate produced the highest level of antibacterial activity in the blood with both the 250-mg and 500-mg doses and maintained the highest concentration up to 8 hr after treatment. Erythromycin stearate produced an activity level almost as high with the 250-mg dose and higher when 500 mg was used. The stearate maintained greater antibacterial activity in sera at the end of 8 hr than did the antibiotic base.

The antibacterial concentrations of the three erythromycin preparations can be explained in part by the recovery rates in the urine-excretion studies. A larger percentage of the total dose of the erythromycin base was extracted (8.3% and 10.6%) in patients receiving 250-mg and 500-mg dosages as compared with the recovery of the propionate (5.6 and 6.8%). The stearate form yielded 5.5% for both dosages. This seems to indicate that the higher blood levels of the propionate and stearate derivatives may be due either to more efficient absorption from the gastrointestinal tract or to a slower rate of excretion by the renal system.

Our observation that the ingestion of food prior to medication with any one of the erythromycin preparations results in lower antibacterial levels in sera, as shown in Table 2 and in Fig. 2 and 4, confirms the reports of Hirsh [6] and Clapper [7].

None of the patients in this study experienced any gastrointestinal disturbance or other side effect attributable to the antibiotics.

CONCLUSION

In the light of our present experience, we conclude that erythromycin propionate and erythromycin stearate achieve higher and more sustained antibacterial effects in the serum than does the antibiotic base in equivalent oral dosages. The higher dose of 500 mg, taken on a fasting stomach, afforded clinically significant antimicrobial activity in the serum.

ACKNOWLEDGMENT

The authors wish to express their gratitude for the assistance in this study rendered by Dr. Augusto J. Felix and Dr. Jose L. Cabezon.

This investigation was supported in part by grants from Eli Lilly and Company, Indianapolis, Indiana and Abbott Laboratories, North Chicago, Illinois.

SUMMARY

In a triple crossover experiment equivalent doses of erythromycin base, erythromycin propionate, and erythromycin stearate were administered orally before breakfast to 60 hospital patients. Antibiotic assays reflected the concentrations in blood sera and the urinary recovery rate.

In 38 patients on a single dose of 250 mg of the three preparations and in 22 who received 500 mg the average serum levels indicated that erythromycin propionate produced the highest activity at 3 hr and maintained the highest level 8 hr after medication. When the stearate form was administered in 500-mg doses its serum activity at 3 hr, and continually until 8 hr, exceeded that of the base.

In a group of ten patients on each dosage the preparations were administered after the morning meal and assays demonstrated that food intake depressed the serum antibiotic activity as compared with levels achieved in blood specimens from fasting patients.

In assays of urine 24 hr after medication, a greater percentage of the total dose of erythromycin base was recovered than was obtained with the propionate or stearate forms.

No untoward reactions to any of the three erythromycin medications occurred during this investigation.

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A CLINICAL AND LABORATORY EVALUATION OF PROPIONYL ERYTHROMYCIN ESTER LAURYL SULFATE IN SOFT TISSUE INFECTIONS

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INTRODUCTION

Since the discovery of erythromycin by McGuire and his associates [1], an ever-increasing literature attests to the clinical effectiveness of this antibiotic in the treatment of many infections caused by Gram-positive bacteria, Gram-negative bacteria, large viruses, and spirochetes. Erythromycin, like other antibiotics such as penicillin and the tetracyclines, after successful clinical and laboratory evaluations in relation to infectious processes has been modified repeatedly in an effort to find a more efficient form of the drug.

It has been demonstrated, that erythromycin esters made with 2-4 carbon chain aliphatic acids produce higher serum concentrations than does the erythromycin base. The most significant increases were found with the propionyl ester [2] and were attributed to its limited excretion through the biliary tract.

At this hospital, in a clinical and laboratory investigation, Marmell [3] found erythromycin propionate effective in the treatment of gonorrhea. Shidlovsky [4] compared the erythromycin base with the proprionate form and erythromycin stearate using a triple crossover technique in an in vivo study of antibiotic serum levels and urinary excretion rates. At 3 and 8 hr after the single oral dose the erythromycin propionate produced higher levels than the other preparations. It was also evident that the ingestion of food prior to the administration of any of these three forms of erythromycin had a depressant effect on the antibacterial activity in blood sera.

To combat the factor of gastric acidity, erythromycin propionate has been further modified. Propionyl erythromycin lauryl sulfate [5] is prepared from erythromycin by esterification followed by a double decomposition reaction with sodium lauryl sulfate to form the salt. This compound has the advantage that it is relatively insoluble in water, tasteless, and stable in gastric secretions. It affords high serum levels of activity whether taken on an empty stomach or administered with food [6]. The high and sustained duration of the antibacterial effect of this erythromycin salt was thought by Anderson [7] and his coworkers to be due in part to its limited biliary excretion and slow rate of disappearance from various tissues.

The chemical, physical and antibacterial properties of propionyl erythromycin lauryl sulfate, its stability in gastric secretions, its palatability, and the high and prolonged antibiotic levels achieved whether taken while fasting or when given with food recommended its clinical trial in soft tissue infections, which are common hospital problems.

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*This investigation was supported, in part, by a research fellowship from Eli Lilly and Company, Indianapolis, Indiana. Their trade name for propionyl erythromycin ester lauryl sulfate is Ilosone Lauryl Sulfate.

MATERIALS AND METHODS

One hundred and six adults, 36 males and 70 females ranging in age from 18 to 65 years, with soft tissue infections requiring definitive therapy, in our out-patient clinics and on hospital wards, were used as subjects.

On the theory that Gram-positive organisms are most frequently encountered in closed infections we selected 68 such cases for treatment with propionyl erythromycin ester lauryl sulfate. These infections were classified by diagnosis as follows: postpartum mastitis, 12 cases; paronychia, 12 cases; hydradenitis suppurativa, 10 cases; carbuncles, 9 cases; felons, 5 cases; abscesses with cellulitis, 7 cases; phlebitis and cellulitis, 4 cases; pilonidal abscesses, 3 cases; suppurative tenosynovitis, 6 cases.

Thirty-eight patients had open infections that drained purulent material. These were diagnosed as follows: lacerated wounds (emergency suture), 12 cases; post-operative laparotomy wounds, 6 cases; second and third degree burns, 8 cases; radical mastectomy wounds, 6 cases; radical neck dissection wounds, 4 cases; thyroidectomy wounds, 2 cases.

For bacteriological study 174 cultures were made. These included follow-up cultures from 30 patients before complete healing had occurred.

Two broth tubes and three plates were employed for these bacteriological determinations. The medium of two plate cultures was tryptose blood agar plus 5% human blood and the third plate used Endo's medium. Tryptophosphate and thioglycollate broths were employed. Sensitivity tests were performed by the disc method on blood-agar plates.

All patients were observed closely and appropriate laboratory procedures were done to correlate clinical findings. At no time was incision and drainage of an abscess delayed when the patient's condition dictated this intervention. When toxic manifestations or resistant organisms were encountered medication was withdrawn.

Doses of 250 mg of propionyl erythromycin lauryl sulfate was administered orally in capsule form four times a day or every six hours. Therapy was continued for a median period of five days but in some cases the courses of treatment extended to 21 days. In cases of postpartum mastitis, patients in lactation received 5 mg of stilbesterol three times daily for five days in conjunction with the antibiotic.

RESULTS

Pertinent data on all cases in this study are presented in Table 1 while Table 2 details the sensitivity of clinical bacterial isolates to five antibiotics.

Postpartum mastitis. Twelve patients, of whom ten were in active lactation, were treated for postpartum mastitis. In all instances the breast was tender, reddened, and usually exhibited a central localized indurated area. Fluctuation was present in only two cases for which immediate incision and drainage was performed. In six cases, spontaneous resolution of the infection occurred during treatment with propionyl erythromycin lauryl sulfate. Four patients required delayed incision and drainage after the infection localized.

Staphylococcus aureus was isolated in six cultures from mammary exudates and the strains recovered were highly susceptible to the antibiotic employed clinically. In two cases erythromycin-sensitive hemolytic streptococci were cultured. The following history is illustrative of the course of patients in this group:

Table 1. Data on 106 Cases of Soft Tissue Infections Treated with Propionyl Erythromycin Lauryl Sulfate

Diagnosis	Total number of patients	Immediate I and D*	Delayed I and D or excision	Sutures removed for drainage	Spontaneous resolution	Therapeutic response	
						Unsatisfactory	Satisfactory
Closed infections							
Postpartum mastitis	12	2	4		6		12
Paronychia	12		8		4		12
Hydradenitis suppurativa	10		6		4	2†	8
Carbuncles	9		7		2		9
Felons	5		5				5
Abscesses with cellulitis	7		1	4	2	1‡	6
Phlebitis and cellulitis	4		1		3		4
Pilondial abscesses	3		3				3
Suppurative tenosynovitis	6		6				6
Open infections							
Lacerated wounds	12			12			12
Postoperative laparotomy wounds	6			6		3	3
Second and third degree burns	8						8
Radical mastectomy wounds	6			6			6
Radical neck dissection wounds	4			4			4
Thyroidectomy wounds	2			2			2

*Incision and drainage
†Dense scar tissue
‡Organisms not susceptible to erythromycin

Table 2. Sensitivity of Bacterial Isolates From Soft Tissue Infections to Five Antibiotics

Bacteria	Erythromycin propionate		Erythromycin base		Vancomycin		Penicillin		Tetracycline	
	S*	R	S	R	S	R	S	R	S	R
Staphylococcus aureus	130	18	130	18	146	2	96	52	110	38
Staphylococcus aureus var. *albus*	18		18		18		14	4	18	
Beta-hemolytic streptococci	14		14		14		14		14	
Alpha-hemolytic streptococci	8		8		8		8		8	
Enterococcus sp.	10		10		10		3	7	3	7

*S = Sensitive
R = Resistant

Case 1 (Hosp. No. 60-6359). A 21-year-old woman, five weeks postpartum, reported to the clinic with a "caked left breast." Her temperature was 99.5 F and there was an indurated, inflamed area over the outer lower quadrant of the left breast which was tender and painful. The patient was lactating and no breast supports could be tolerated because of the discomfort. Propionyl erythromycin lauryl sulfate and stilbesterol were prescribed and the patient returned in three days with normal temperature. She reported complete relief of pain in the affected breast. Some milk could still be expressed from the nipple but the breast was soft and the formerly inflamed area showed slight induration but was no longer erythematous or tender. The patient was seen again after four days and the pathology had abated completely.

Paronychia. Twelve patients with paronychia were seen in the out-patient clinic with infections in varying stages of the suppurative process. With propionyl erthromycin lauryl sulfate medication and warm saline soaks four of these infections resolved without surgery and eight patients ultimately required incision and drainage. Healing thereafter was uncomplicated. *S. aureus* was present in all cultures and was found to be highly susceptible to the antibiotic.

Hydradenitis suppurativa of the axillae. This diagnosis was established in three patients with bilateral involvement and the remaining seven had hydradenitis suppurativa of one axilla. After antibiotic therapy spontaneous resolution of the infection occurred in four patients while six required incision and drainage of the affected areas. In two patients the chronicity of the infection necessitated excision of indurated suppurative areas that had ruptured spontaneously and formed chronically draining sites.

Cultures from two patients grew pure colonies of *S. albus* that were sensitive to propionyl erythromycin lauryl sulfate. In two cases drainage persisted over a period of four weeks and required excision of the entire axillary surface tissue despite antibiotic sensitivity of the *S. aureus* cultured from three follow-up specimens and what was considered adequate antibiotic dosage.

Posttreatment healing in all other cases was satisfactory.

Carbuncles. Of nine cases under study, two resolved with medication and warm soaks. Seven patients required incision and drainage. *S. aureus* was found in all cultures and proved to be sensitive to the propionyl erythromycin lauryl sulfate. The wounds healed without complication.

Felons. Five cases of felons all required incision and drainage. The sensitivity of the recovered *S. aureus* demonstrated in vitro was reflected in prompt regression of pain and swelling and satisfactory healing.

Abscesses with cellulitis. Seven patients with such infections received propionyl erythromycin lauryl sulfate. Local pyoderma with cellulitis in two patients healed spontaneously. Four patients in this group had sutured lacerated wounds of the extremities with local abscess formation, lymphangitis, and lymphadenitis. These responded after removal of the sutures, manual wound dehiscence, and antibiotic therapy.

With one exception, clinical isolates in these patients were hemolytic streptococci which were sensitive to the antibiotic. A susceptible strain of S. aureus was also demonstrated in one patient.

The resistant strain of S. aureus was disclosed in the case of a woman with an abscess of the face with localized peripheral cellulitis. Because of the recalcitrance of this infection to commonly available antibiotics, this case is presented in detail.

Case 2 (Hosp. No. 60-2971). A 32-year-old housewife accidentally scratched her face with the bristles of a hairbrush. In 24 hr there was pain and tenderness in the area. She reported to the outpatient clinic approximately 36 hr after the incident.

An indurated, tender, inflamed area about 2 in. in diameter was present over the right malar eminence. The patient was given propionyl erythromycin lauryl sulfate and instructed to return in 2 days

On the second visit there was a marked increase in the inflammatory area involving almost the entire right face with edema of the right eyelid, and temperature elevation to 103.6 F. The patient was hospitalized and, when the area of primary involvement became fluctuant, incision and drainage was performed.

The incision drained purulent material that cultured S. aureus resistant to propionyl erythromycin lauryl sulfate, vancomycin, penicillin, and the tetracyclines, but proved susceptible to kanamycin.

Warm saline soaks and therapy with kanamycin were instituted, and the patient made a rapid and uneventful recovery.

Phlebitis and cellulitis. Included in this study were four drug addicts who reported to the clinic for pain and swelling originating at sites of narcotic injections. Phlebitis of antecubital veins and cellulitis were present in all cases. The infections in three patients regressed after treatment with propionyl erythromycin lauryl sulfate and one patient required incision and drainage. Subsequent healing was without incident.

Erythromycin-sensitive enterococci were found in two cases and in one a susceptible strain of S. aureus.

Pilonidal abscesses. Our three cases of pilonidal abscesses all required incision and drainage, but healed satisfactorily after this procedure combined with antibiotic therapy. Erythromycin-sensitive S. aureus was cultured, but pathogenic Gram-negative organisms were present in all these cases.

Suppurative tenosynovitis. In our surgical clinics we encountered six cases of suppurative tenosynovitis of the hand and all patients were promptly hospitalized. Four cases required incision and drainage and in two patients the original traumatic wounds were surgically enlarged for adequate drainage. All organisms cultured from these patients were Gram-positive and proved sensitive to propionyl erythromycin lauryl sulfate. The following is a typical case:

Case 3 (Hosp. No. 60-3946). A 32-year-old male sustained a through-and-through ice-pick wound on the lateral aspect of the left index finger between the proximal and distal phalanges. No fracture was disclosed on x-ray examination. The wound was irrigated with normal saline, a dressing with splint applied, and tetanus antitoxin administered.

When the patient was seen again after 48 hr he complained of severe pain in the finger, which was markedly swollen and incapable of flexion. The wound was suppurating and draining only moderately. The patient's temperature was 101.6 F. The white blood cell count was 12,000 with 78% polymorphonuclear cells.

A diagnosis of tenosynovitis was made and the wound was enlarged with a hemostat. Warm saline soaks and propionyl erythromycin lauryl sulfate were prescribed. Cultures revealed S. aureus and enterococci sensitive to the antibiotic and vancomycin but resistant to penicillin and the tetracyclines.

In 48 hr after medication was instituted the swelling subsided and drainage from the wound ceased. There was complete defervescence and the wound healed progressively thereafter.

Sutured lacerated wounds of face and scalp. Twelve patients who had had emergency suture of lacerations five days prior to their clinic visit were found to have infected wounds draining purulent material and exhibiting varying degrees of peripheral cellulitis.

With suture removal, warm saline soaks, and therapy with propionyl erythromycin lauryl sulfate, the infections subsided and all wounds granulated and healed normally.

Erythromycin-sensitive enterococci were present in three of these cases and susceptible staphylococci and sometimes streptococci were recovered in the other cases.

Postoperative laparotomy wounds. The laparotomy wounds of six patients subjected to emergency exploration were potentially contaminated initially and later were obviously infected. Three of these demonstrated S. aureus resistant to propionyl erythromycin lauryl sulfate, including one strain also resistant to vancomycin. The other cases represented infections mixed with the coliform group. Therapy with propionyl erythromycin lauryl sulfate was instituted in each patient several days before bacteriological reports were available. During this period there was no evidence of control of the infection. Consequently the wounds were widely opened and packed and treatment with another antibiotic substituted, with subsequent normal healing in all cases.

Second- and third-degree burns. All Gram-positive organisms cultured from eight patients who had suffered second- and third-degree burns were sensitive to propionyl erythromycin lauryl sulfate. However, mixed infections were present in all cases. Infected areas went on to slough and later required debridement.

No antibiotic can be expected to eliminate bacteria from devitalized tissue such as occurs in burns. However, the appropriate antibiotic may be useful in controlling the spread of infection from such a focus. In such cases either enzymatic or surgical debridement should be employed to eliminate necrotic tissues.

Radical mastectomy wounds. In six cases in which infection supervened after radical mastectomy, the skin flaps were found to be too tight and slough occurred at the suture sites. S. albus sensitive to propionyl erythromycin lauryl sulfate was isolated in four cases and sensitive S. aureus in two cases.

All patients improved under medication with propionyl erthromycin lauryl sulfate but in two cases the slough was attributed to tension necrosis rather than to infection per se.

Radical neck dissection wounds. In four patients with infections in wounds following radical neck dissections there was prompt response to propionyl erythromycin lauryl sulfate, both clinically and bacteriologically.

Thyroidectomy wounds. Infection was evident in incision wounds in two patients after thyroidectomy. Erythromycin-sensitive staphylococci were present in both cases. One wound healed dramatically under therapy with propionyl erythromycin lauryl sulfate and the other resolved after five days following the evacuation of a hematoma.

Side effects. Three patients exhibited symptoms that could be related to drug toxicity, all involving gastrointestinal disturbances. One patient who received propionyl erythromycin lauryl sulfate for fourteen days developed diarrhea that subsided after withdrawal of the medication. Bacteriological study of fecal specimens did not demonstrate any overgrowth of intestinal staphylococci. Two patients complained of nausea without vomiting; one after twenty-three days of antibiotic therapy and the other after seven days. These complaints also subsided when the medication was withdrawn. The three patients whose treatment was necessarily interrupted because of side effects ultimately required further surgery to correct the infections in open wounds draining purulent material.

No deaths occurred in this series, nor did laboratory studies indicate any deleterious effect on systems other than the mild gastrointestinal disorder that affected less than 3% of the patients.

Bacteriology. Cultures were made from clinical exudates from 80 of the 106 patients under this investigation. In the remaining cases, material for bacteriological culture was not available since the closed infections resolved spontaneously. The following Gram-positive microbiological strains were isolated: Staphylococcus aureus, 148 strains; Staphylococcus aureus var. albus, 18 strains; beta-hemolytic streptococci, 14 strains; alpha-hemolytic streptococci, 8 strains; enterococci, 10 strains.

Strains of the following Gram-negative organisms were also isolated: Proteus sp., Escherichia coli, Pseudomonas aeruginosa, Alcaligenes faecalis, and paracolon bacillus.

Follow-up studies increased the total number of cultures of 174. Sensitivity determinations of clinical isolates to five antibiotics are recorded in Table 2. It was found that 130 strains of S. aureus, of a total of 148, were susceptible to propionyl erythromycin lauryl sulfate.

DISCUSSION

Propionyl erythromycin lauryl sulfate, a new salt of erythromycin, has demonstrated in animal experiments and in vivo higher serum activity than other forms of erythromycin on equivalent oral dosages.

We used this new antibiotic in the treatment of infections in 106 adults. Sixty-eight of these patients, at the onset of therapy, had closed infection and 38 had purulent open wounds. The medication produced spontaneous remission in many infections without the necessity for incision and drainage. Where surgery was indicated it was of only minor degree.

Failure of clinical response corresponded with findings of erythromycin-resistant organisms in four cases, three of infected laparotomy wounds, and one of cellulitis of the face. Staphylococci resistant to vancomycin were isolated from two cases. Other resistant staphylococci were found only in follow-up cultures where medication had been given over a period of at least twelve days and these cases ultimately required further surgery to bring the infection under control.

Side reactions were mild and infrequent, occurring as gastrointestinal complaints in only three cases.

Where S. aureus organisms resistant to erythromycin were encountered there was correlation in the clinical behavior of the infection. The other resistant organisms made their appearance generally after prolonged treatment of suppurative open wounds which actually required further surgery, such as debridement, wide open drainage, or removal of nonabsorbable suture material.

The antibiotic proved ineffective in laparotomy wounds where resistant staphylococci were found and also where mixed infections with the coliform group supervened. The drug proved highly effective in comparison with penicillin or the tetracyclines in the so-called "fairly clean" wounds, such as previously sutured lacerations from which enterococci were cultured.

CONCLUSION

Propionyl erythromycin lauryl sulfate was well tolerated and was effective both clinically and bacteriologically against organisms found in the antimicrobial spectrum of erythromycin, with few resistant strains developing in our study.

This new ester of erythromycin is an excellent adjunct in the surgical management of soft tissue infections.

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DISCUSSION

DR. McCORMICK (Eli Lilly): Dr. Morton, what was the level of resistance for vanocomycin that you reported?

DR. MORTON: We didn't bring that out in the paper.

DR. McCORMICK: Was it high or low?

DR. MORTON: It was quite high.

DR. ANDERSON: I would like to know what the criterion was for susceptibility and resistance of the organisms that you tested here.

DR. MORTON: We used the disc method. This was done by the bacteriology lab. We left determinations of resistant and nonresistant up to them.

DR. PRIGOT: We accept any organism as being susceptible that is susceptible to the amount of antibiotic that we can detect in the sera. In other words we take the patient's serum and use it in the cup plate method against a specific organism for that patient; we know that the patient's serum has some antibiotic in it and that's all. And anything above this is resistant and anything below is susceptible.

DR. ANDERSON: Would that be above or below the concentrations that you previously showed on the chart?

DR. PRIGOT: That would be above.

DR. ANDERSON: You mean, it is susceptible if it is above – or do you mean resistant? Then your 1.2 μg would be significant also in the other instance. And do increments of 0.2 μg makes a difference in susceptibility?

DR. PRIGOT: Yes, I would like to ask anybody who has a strain resistant to vancomycin that they forward it to me. We have gone through over 5000 strains of Staphylococcus aureus and have not found any resistance to vancomycin. Of those that have been submitted to us as resistant a number have been contaminated with a Gram-negative organism. And I can see how this can occur. Some of the people who have submitted these have been Ph.D's in bacteriology and I don't think you need to be too embarrassed if you send such a culture to us because if a smear is made and observed for Gram-positive organisms, there can be a few Gram-negatives in the background. When these are plated on a culture plate and a disc used, the Gram-negatives can grow out and show resistance around this disc. We have also found some discs that don't have antibiotic in them. This has been rare, thank goodness, for the past year or so.

COMPARISON OF ANTIBACTERIAL ACTIVITY IN THE SERA OF SUBJECTS INGESTING PROPIONYL ERYTHROMYCIN AND PROPIONYL ERYTHROMYCIN LAURYL SULFATE

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INTRODUCTION

Recently we reported that there was greater antimicrobial activity in the serum following ingestion of the pediatric preparation of propionyl erythromycin lauryl sulfate* than with erythromycin ethyl carbonate [1]. The propionyl erythromycin lauryl sulfate pediatric preparation has been established as an effective antibiotic in the treatment of bacterial infections [2, 3]. This present paper compares the antibacterial activity in the serum from subjects who have ingested propionyl erythromycin lauryl sulfate and propionyl erythromycin.

METHODS

Both the lauryl sulfate salt of the propionyl erythromycin ester and propionyl erythromycin ester† used were commercially available capsules each containing 250 mg of erythromycin activity.

Ten healthy adults received each of the two preparations while fasting, i.e., food was not permitted until after the 2-hr blood specimen was obtained. The dosage schedule was 250 mg every 6 hr for three doses. A typical crossover-type experiment was set up. The ten subjects were divided into two groups, A and B. Group A took antibiotic No. 1 and Group B, antibiotic No. 2. The study was then repeated but the medication was reversed so that Group A took antibiotic No. 2 and Group B, antibiotic No. 1. Two days were allowed between the two halves of the study so that there was no carry-over.

One week later the above study was repeated with the same ten subjects. However, for this portion of the experiment the three doses of antibiotic were administered at the time each subject received his meal. The diet was identical for all ten subjects.

Blood samples were drawn from each subject before and at $\frac{1}{2}$, 1, 2, 4, and 6 hr after taking the first dose, and at 1, 2, 4, and 6 hr after the second and third doses. These samples were centrifuged and the sera were assayed on the same day on which they were obtained by a modification of the Rammelkamp serial twofold tube-dilution method [4]. A strain of beta-hemolytic Streptococcus (C203) was used as the indicator organism.

Jones [5] and Perry [6] have recommended the use of the tube-dilution method for comparing effectiveness of antibiotics in patients' sera. Our results are reported as the reciprocal of the dilution of the serum, i.e., the number of times the serum can be diluted and still inhibit the bacterial pathogen. This may also be interpreted as the number of times more antibiotic there is in the serum than is actually required to inactivate or inhibit the pathogen.

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*The trade name of Eli Lilly and Company for propionyl erythromycin ester lauryl sulfate is Ilosone.

†The propionyl ester of erythromycin will not be available commercially after September, 1960.

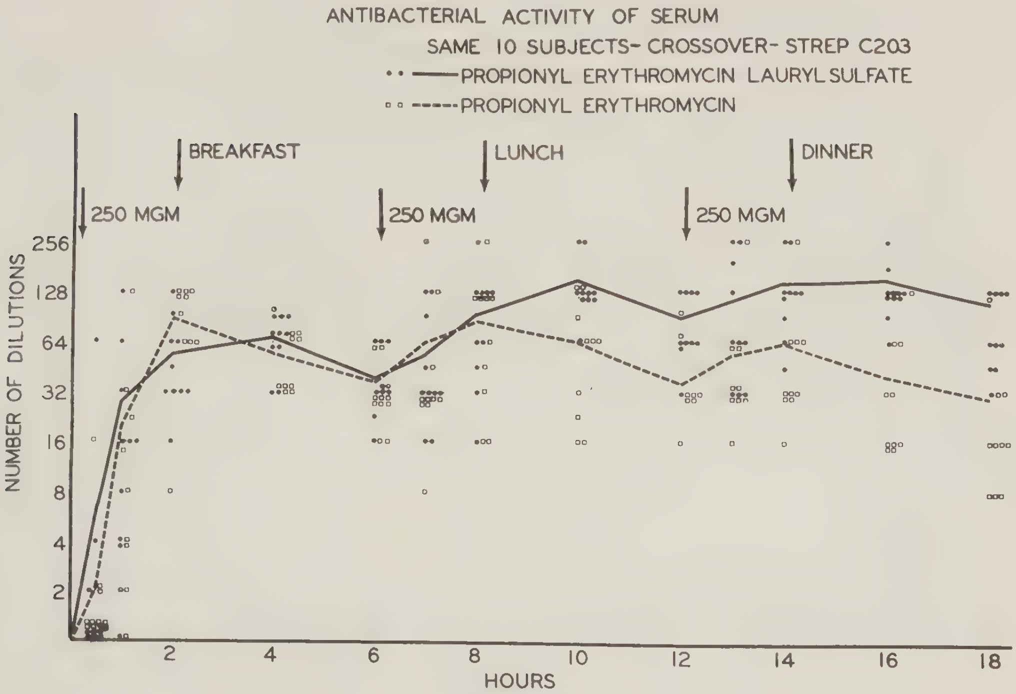
ANTIBACTERIAL ACTIVITY OF SERUM
SAME 10 SUBJECTS- CROSSOVER- STREP C203
• • —— PROPIONYL ERYTHROMYCIN LAURYL SULFATE
▫ ▫ ----- PROPIONYL ERYTHROMYCIN
BREAKFAST
LUNCH
DINNER
250 MGM
250 MGM
250 MGM
NUMBER OF DILUTIONS
256
128
64
32
16
8
4
2
2
4
6
8
10
12
14
16
18
HOURS

Fig. 1

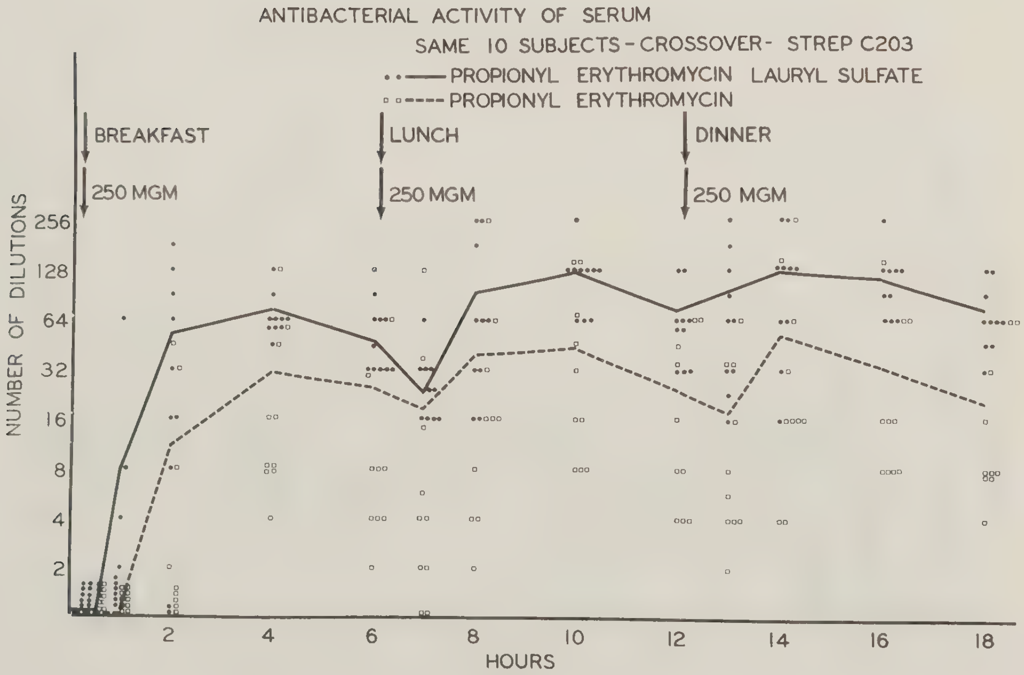
ANTIBACTERIAL ACTIVITY OF SERUM
SAME 10 SUBJECTS - CROSSOVER- STREP C203
• • —— PROPIONYL ERYTHROMYCIN LAURYL SULFATE
▫ ▫ ----- PROPIONYL ERYTHROMYCIN
BREAKFAST
LUNCH
DINNER
250 MGM
250 MGM
250 MGM
NUMBER OF DILUTIONS
256
128
64
32
16
8
4
2
2
4
6
8
10
12
14
16
18
HOURS

Fig. 2

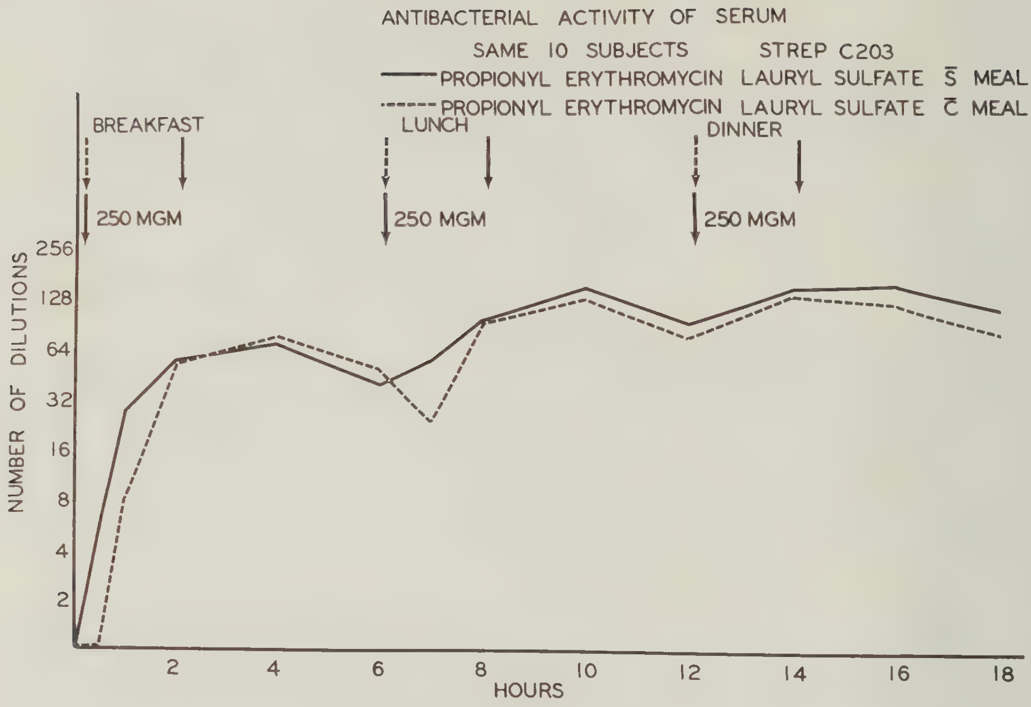
ANTIBACTERIAL ACTIVITY OF SERUM
SAME 10 SUBJECTS STREP C203
PROPIONYL ERYTHROMYCIN LAURYL SULFATE S̄ MEAL
PROPIONYL ERYTHROMYCIN LAURYL SULFATE C̄ MEAL
BREAKFAST
LUNCH
DINNER
250 MGM
250 MGM
250 MGM
NUMBER OF DILUTIONS
256
128
64
32
16
8
4
2
2
4
6
8
10
12
14
16
18
HOURS

Fig. 3

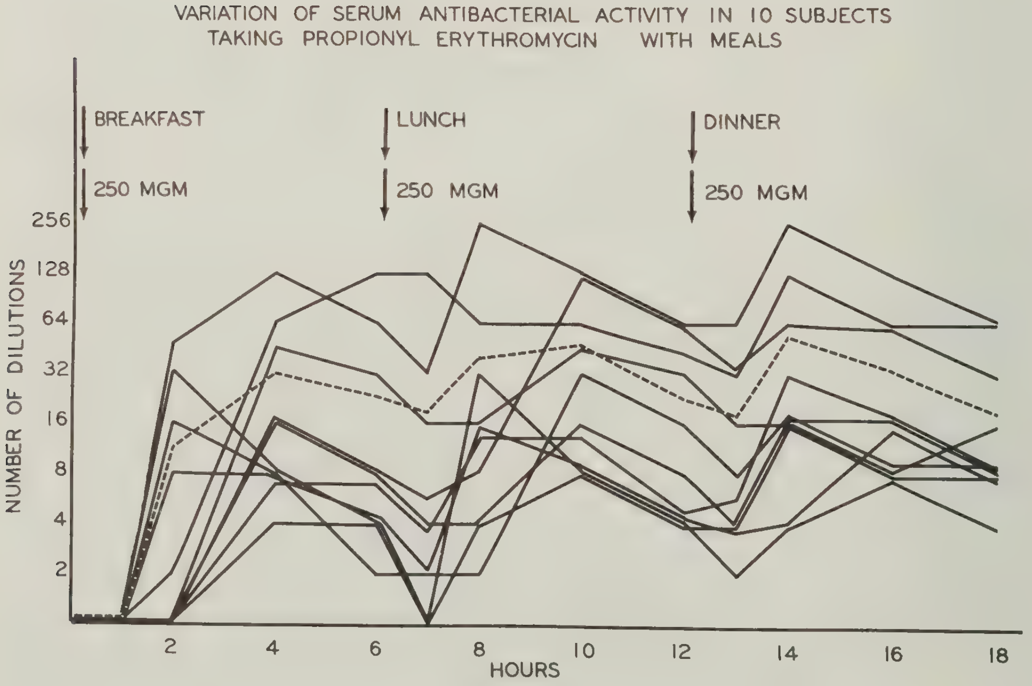
VARIATION OF SERUM ANTIBACTERIAL ACTIVITY IN 10 SUBJECTS
TAKING PROPIONYL ERYTHROMYCIN WITH MEALS
BREAKFAST
LUNCH
DINNER
250 MGM
250 MGM
250 MGM
NUMBER OF DILUTIONS
256
128
64
32
16
8
4
2
2
4
6
8
10
12
14
16
18
HOURS

Fig. 4

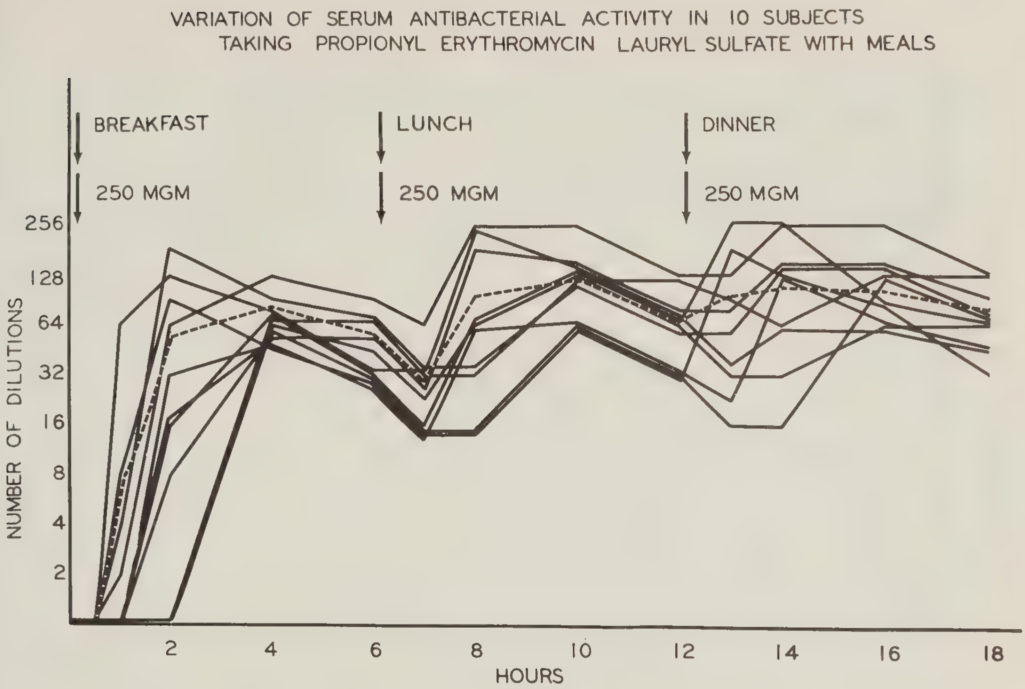
VARIATION OF SERUM ANTIBACTERIAL ACTIVITY IN 10 SUBJECTS
TAKING PROPIONYL ERYTHROMYCIN LAURYL SULFATE WITH MEALS
BREAKFAST
LUNCH
DINNER
250 MGM
250 MGM
250 MGM
NUMBER OF DILUTIONS
256
128
64
32
16
8
4
2
2
4
6
8
10
12
14
16
18
HOURS

Fig. 5

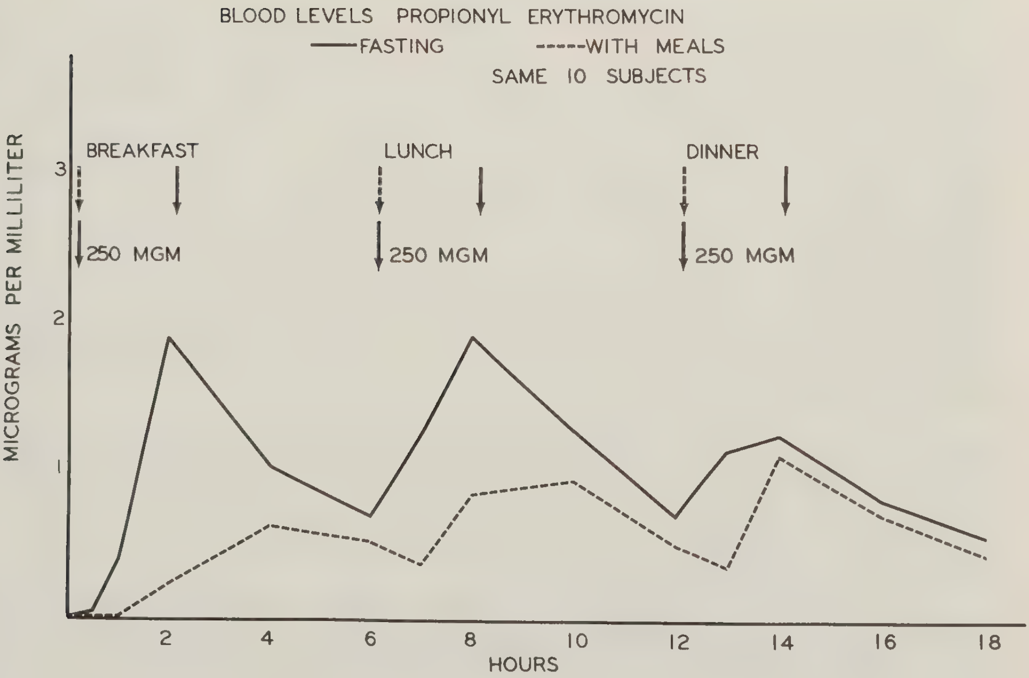
BLOOD LEVELS PROPIONYL ERYTHROMYCIN
——FASTING
-----WITH MEALS
SAME 10 SUBJECTS
BREAKFAST
LUNCH
DINNER
250 MGM
250 MGM
250 MGM
MICROGRAMS PER MILLILITER
3
2
1
2
4
6
8
10
12
14
16
18
HOURS

Fig. 6

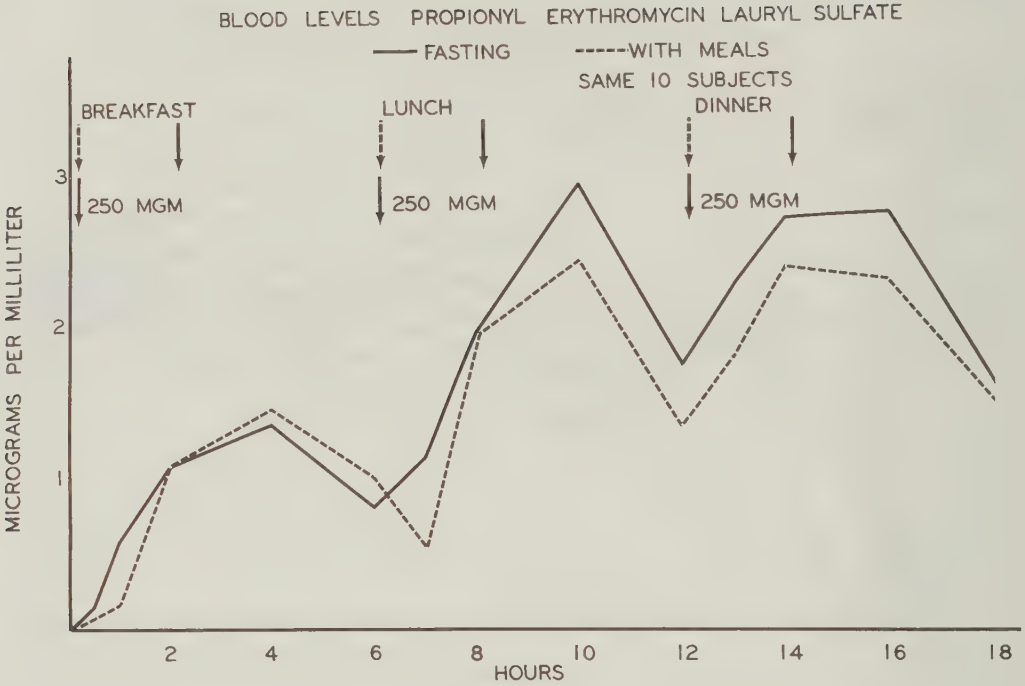

Fig. 7

RESULTS

The data have been summarized in Figs. 1, 2, and 3. Two important facts are observable. The antibacterial activity in the serum following ingestion of the lauryl sulfate salt of propionyl erythromycin is as rapid in onset, reaches equally high peak values, and is of as prolonged activity as that obtained with propionyl erythromycin when both are given while fasting (Fig. 1). As Hirsch and Finland reported [7] we also observed a falling off of antibacterial activity when propionyl erythromycin was given to the subjects with their meals (Fig. 2). This antibacterial activity in the serum of subjects ingesting propionyl erythromycin ester with food is comparable to the activity found following administration of the older forms of erythromycin administered while fasting [8, 9]. Unlike the case of propionyl erythromycin ester, the peak values following administration of propionyl erythromycin lauryl sulfate with meals are similar to those when it is given while fasting (Fig. 3). Individual results have been listed in Tables 1 and 2 for closer scrutiny.

Variation of absorption between individuals was not great when both preparations were given while fasting. However, when they were administered with food there was apparently a marked difference in each individual's ability to absorb propionyl erythromycin. This range of variation is illustrated in Fig. 4. The absorption of the lauryl sulfate form administered with food was remarkably consistent for the ten test subjects (Fig. 5).

Figures 6 and 7 illustrate the average blood levels for propionyl erythromycin ester and propionyl erythromycin lauryl sulfate, with and without food. A linear method of depicting the results in micrograms makes the differences between the preparations with and without food more apparent.

Table 1. Comparison of the Antibiotic Activity of Propionyl Erythromycin and Propionyl Erythromycin Lauryl Sulfate, Without Food

Time, hr	Patient										
	1	2	3	4	5	6	7	8	9	10	Avg
Propionyl erythromycin											
0	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
250 mg											
½	<2	<2	2	<2	2	16	<2	<2	<2	<2	<2
1	<2	4	24	2	32	128	16	2	8	4	22
2	128	128	128	64	64	128	96	64	128	8	94
Food											
4	96	32	64	32	32	64	32	32	64	64	51
6	64	32	64	16	32	32	16	32	32	32	35
250 mg											
1	32	48	32	8	128	256	32	32	32	32	63
2	128	64	48	16	128	256	32	128	16	128	94
Food											
4	128	64	32	16	128	96	16	64	24	64	63
6	96	32	32	8	64	32	8	32	16	32	35
250 mg											
1	256	32	32	64	64	32	16	32	8	32	57
2	256	32	32	32	64	32	16	64	32	64	62
Food											
4	128	32	16	16	32	16	16	64	16	64	40
6	128	16	16	16	32	8	8	32	8	16	28
Propionyl erythromycin lauryl sulfate											
0	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
250 mg											
½	2	<2	<2	<2	<2	64	<2	<2	4	<2	7
1	64	16	4	16	16	128	32	2	8	4	29
2	32	16	64	96	32	128	48	32	32	64	54
Food											
4	96	32	64	64	64	64	96	32	96	64	67
6	64	24	32	32	32	16	64	32	64	32	39
250 mg											
1	128	16	32	32	32	86	32	16	128	48	56
2	256	16	128	64	128	64	48	32	128	128	99
Food											
4	256	128	128	128	128	64	128	256	128	128	147
6	128	64	64	64	64	32	128	128	128	64	86
250 mg											
1	192	32	64	32	128	32	256	256	64	64	112
2	256	48	128	96	128	64	256	128	128	128	136
Food											
4	256	96	192	128	128	64	128	128	128	128	138
6	128	48	64	64	48	32	128	128	128	64	83

Table 2. Comparison of the Antibiotic Activity of Propionyl Erythromycin and Propionyl Erythromycin Lauryl Sulfate, With Food

Time, hr	Patient										
	1	2	3	4	5	6	7	8	9	10	Avg
Propionyl erythromycin											
0	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Food + 250 mg											
½	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
1	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
2	2	<2	<2	<2	16	32	8	48	<2	<2	11
4	64	4	48	16	8	8	8	128	8	16	31
6	128	4	32	8	2	4	4	64	8	8	26
Food + 250 mg											
1	128	2	16	4	2	2	2	32	4	6	19
2	64	4	16	4	2	32	16	256	16	8	42
4	64	8	48	16	32	8	8	128	16	128	46
6	48	4	32	8	16	4	4	64	8	64	25
Food + 250 mg											
1	32	4	16	4	8	2	4	64	6	32	17
2	128	16	16	16	16	4	4	256	32	64	55
4	64	8	8	16	8	8	16	128	16	64	34
6	64	8	16	8	8	4	8	64	8	32	22
Propionyl erythromycin lauryl sulfate											
0	4	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Food + 250 mg											
½	4	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
1	2	<2	<2	<2	64	<2	8	4	<2	<2	8
2	64	<2	<2	16	128	8	96	192	16	32	55
4	128	64	64	64	64	64	48	96	64	64	72
6	96	48	64	32	32	32	32	64	64	32	50
Food + 250 mg											
1	64	24	32	16	32	16	16	32	24	16	27
2	256	64	32	64	256	16	16	37	192	64	99
4	256	128	128	128	128	64	64	128	128	64	122
6	128	64	128	64	64	32	32	64	64	32	67
Food + 250 mg											
1	128	32	96	256	32	16	192	64	64	24	90
2	256	32	64	256	64	16	128	128	128	128	120
4	256	64	128	96	64	128	96	128	128	64	115
6	128	64	128	64	48	64	32	64	96	48	74

DISCUSSION

The propionyl ester of erythromycin has been shown to have an earlier onset and higher and more prolonged levels than other forms of erythromycin [6-16]. However, like erythromycin base, propionyl erythromycin is destroyed by acids. Like acid-susceptible penicillin G, previous erythromycin preparations produced better absorption when given while fasting [7] and we recommend that for optimal absorption the propionyl ester should be taken while fasting [17].

Recently Stephens et al. [18] reported that the lauryl sulfate of the propionyl ester was acid insoluble and alkaline dissociable (Fig. 8). This insolubility in acid, besides making the preparation highly palatable and easily manufactured as a pharmaceutically elegant pediatric suspension, enables it to pass intact through the acids of the stomach in the presence or absence of food and dissociate in the alkaline contents of the duodenum where it is absorbed as propionyl erythromycin.

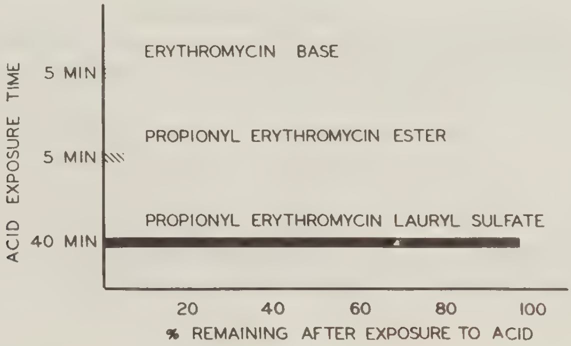

Fig. 8. Effect of gastric acid, pH 1.2.

Studies of Hirsch and Finland [19] and Griffith [1] show that the blood levels with and without food are practically identical following administration of propionyl erythromycin lauryl sulfate. Usually food delays the onset of antibiotic blood levels, lowers the peak of activity, and prolongs the duration of antibiotic concentration of acid-stable antibiotics. A unique feature of propionyl erythromycin lauryl sulfate is that its antibiotic activity peak levels and duration of action in the presence of food are very similar to those which are observed in fasting subjects. This makes it an ideal preparation to give with meals. It can also be given with food, without fear of diminished absorption or therapeutic effectiveness, to decrease the gastrointestinal side effects sometimes associated with the administration of oral antibiotics.

There is a distinct tendency for the propionyl erythromycin lauryl sulfate blood levels to reach higher concentrations with the second and third doses, whereas the propionyl erythromycin blood levels show a slight decline. This is probably a reflection of absorption of the repeated dosage with erythromycin still present in the sera following the first dose, plus the effect of food on absorption. Any food remaining in the stomach when the second and third doses were administered would tend to decrease the absorption of the propionyl erythromycin form, but would not affect the absorption of propionyl erythromycin lauryl sulfate.

The prolonged duration of erythromycin activity in the blood stream suggests that propionyl erythromycin lauryl sulfate could be given less frequently than every 6 hr in infections that are relatively easy to control. In fact once a severe infection has been brought under control with the usual 6-hr dosage schedule, we frequently administer propionyl erythromycin lauryl sulfate with meals and with a bedtime snack (q.i.d.) or even lower the dosage to an 8-hr schedule.

Chemical and paper-chromatographic studies on the sera of subjects who have ingested propionyl erythromycin lauryl sulfate indicate that the activity present is primarily due to propionyl erythromycin. This predominance of the ester is also found in the urine. Approximately 2.5-10% of the orally administered erythromycin is found in the first 6-8 hr after single or repeated doses of propionyl erythromycin lauryl sulfate [8, 20].

Hammond [21] has recently shown clinically (verifying Lee's animal studies [22]) that propionyl erythromycin is excreted less rapidly than erythromycin base via the liver. Hammond has also found that after oral administration, the lauryl sulfate salt of propionyl erythromycin is slowly excreted by the biliary systems like the ester.

The in vitro antibacterial activity of propionyl erythromycin lauryl sulfate is identical to that of propionyl erythromycin [23]; the blood levels of fasting subjects are comparable; chemical and paper-chromatographic studies indicate that the erythromycin ester in the sera of the test subjects has been absorbed free from the lauryl sulfate salt. One can conclude that propionyl erythromycin lauryl sulfate and propionyl erythromycin effectively are the same, except for the acid stability of the former. The acid stability permits the lauryl sulfate form to be given with food and still produce antimicrobial activity equal to that when given while fasting. Thus it is reasonable to substitute the lauryl sulfate salt of propionyl erythromycin when propionyl erythromycin would ordinarily be indicated.

CONCLUSIONS

The total antibacterial activity in the serum of subjects taking repeated doses of propionyl erythromycin lauryl sulfate is greater than the activity obtained with propionyl erythromycin when both are taken on an empty stomach. The propionyl erythromycin ester is not acid stable and produces lower antibacterial concentrations in the serum when taken with food than when fasting. The lauryl sulfate salt of the propionyl erythromycin ester is acid stable and is not susceptible to the destructive action of gastric juice. Unlike the propionyl form of erythromycin, propionyl erythromycin lauryl sulfate achieves essentially as great antibacterial activity in the serum when taken with food as when taken while fasting. Since the antibacterial action of the lauryl sulfate salt of propionyl erythromycin is as effective in sera when taken with food as when ingested while fasting, it can be given without regard to meals. More important, however, it can be given with food to decrease gastric intestinal side effects, sometimes seen in susceptible individuals, without fear of decreased absorption and can achieve the desired clinical effectiveness.

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DISCUSSION

FRANK WILK (Merck): I would just like to ask Dr. Griffith if in his stability studies he had his concentrations such that all of these things were in solution?

DR. GRIFFITH: This material was placed in acid under the same conditions that it is placed in the human stomach. This is in the form that we give it in the stomach as concentrated material. This would not be dissolved when placed in acid conditions and it never dissociates in acid, so it would remain stable probably indefinitely.

COMPARATIVE CLINICAL AND EXPERIMENTAL INVESTIGATIONS CONCERNING TISSUE AND SERUM CONCENTRATIONS OF DEMETHYLCHLORTETRACYCLINE AND PYRROLIDINOMETHYLTETRACYCLINE UNDER PATHOLOGICAL CONDITIONS IN MAN

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INTRODUCTION

Until now the only way to determine the therapeutic efficiency of antimicrobial agents was by experimental investigations of animals (tissue and serum) or by determining the concentrations in the serum of man under normal conditions. This does not seem to give a picture of the real situation.

Concentration of a substance in blood or serum is a complex figure. It serves only as an expression of the transport function of blood and serum to bring the substances to the desired location. Therefore, it doesn't necessarily give any precise information about the therapeutic effect. One has to take into consideration certain factors; the absolute concentration in blood or serum of antibiotic substances tells very little about their chemotherapeutic effectivity because there are considerable differences in the antimicrobial activity of these drugs and it is obvious that there is a specific threshold value for every antibiotic. This is true for investigations under physiological conditions and much more so under pathological conditions.

Blood and serum levels depend on dose, resorption, distribution, diffusion in tissue and body fluids, changes in chemical structure, chemical degradation, rediffusion from the tissues, and elimination by kidneys and intestines. To evaluate the therapeutic activity of an antibiotic substance one has to know its specific antibacterial spectrum, its physicochemical properties, the conditions at the desired location of effectivity, and the conditions of chemical alterations. Generally, it may be assumed that more effective substances require a lower concentration in blood or serum.

The discrepancies in the results of various investigations are due to differences in technique and the individual differences of patients themselves (body weight, differences in the speed of resorption, distribution, and elimination under the specific conditions prevailing in disease). Therefore the blood, serum, or tissue levels of various patients cannot be compared without regard to these differences.

Contrary to former assumptions, there is no direct relation between the blood or serum level and the tissue level at the desired location. This has been proved by clinical experience.

Since determinations of serum and blood levels have been done almost exclusively in animal experiments under normal conditions, it seemed worthwhile to perform investigations in sick people. This would tend to correspond more closely to the actual pathological conditions and the results would present better proof of the effects of antibiotic substances.

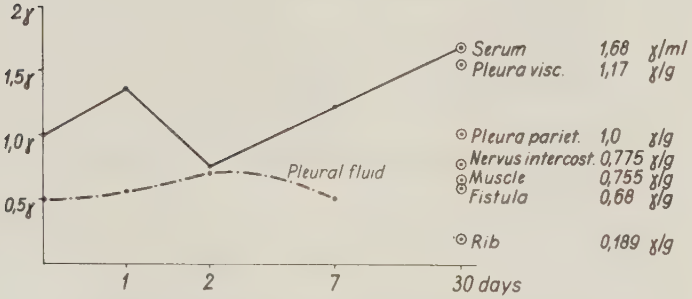

Fig. 1. Serum and tissue concentrations of DMCT (given orally, 600 mg/day, for approx. 30 days) in a 36-year-old male with a bronchopleural fistula and thoracic empyema resulting from a gunshot wound of the thorax.

MATERIALS AND METHODS

DMCT,* 600 mg/day and PMT,† 250 mg/day, were given to 50 patients in whom a preoperative chemoprophylaxis seemed indicated. The serum level was determined before administration of the drug. Serum and tissue samples were taken at operation and thereafter tested simultaneously.

Blood and tissue levels were determined by the cylinder-plate test of Grove and Randall. The test organism used was Bacillus cereus PCI 213/FDA. The blood samples were diluted with pH 4.5 phosphate buffer and the tissue samples with pH 6.8, phosphate buffer. The sensitivity of the test is in the neighborhood of 0.1 μg/ml.

RESULTS AND CONCLUSIONS

The results obtained thus far are based on investigations of 33 patients who were given DMCT and 17 patients who received PMT. A few examples

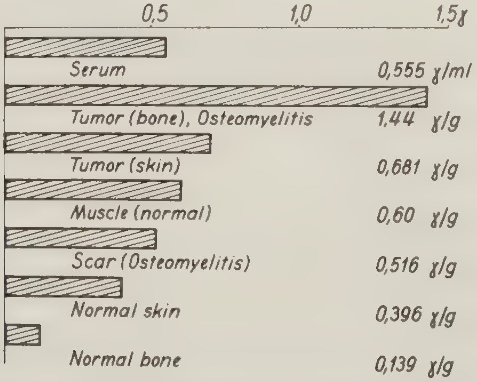

Fig. 2. Serum and tissue concentrations DMCT (given orally, 600 mg/day, for approx. 4 days) in a 36-year-old male with squamous cell carcinoma.

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*Demethylchlortetracycline.
†Pyrrolidinomethyltetracycline.

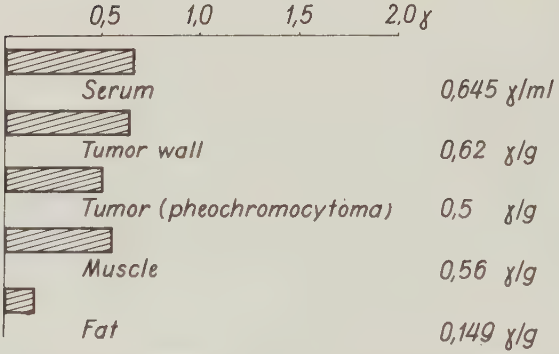

Fig. 3. Serum and tissue concentrations of PM (250 mg/day, i.v., for 3 days) in a 56-year-old male with a pheochromocytoma.

follow. The first example is a case of pleural empyema caused by a bronchopleural fistula resulting from a gunshot wound of the thorax. Thoractotomy, lobectomy of upper lobe which contained the fistula, and local pleurectomy were performed. DMCT levels in various tissue samples show considerable differences. One cannot detect a direct relation between the serum and tissue levels. There is a strikingly high tissue level in the visceral pleural scar. This was the zone of the strongest inflammatory reaction.

A second example is the case of a 36-year-old male Egyptian who had undergone surgery for squamous cell carcinoma of the right lower jaw and had to be reoperated because of a local recurrence of the disease. The data are shown in Fig. 2.

Again one is struck by the differences in DMCT levels in various tissue samples. High concentrations are found in the bone of the lower jaw, which showed considerable pathological changes. It is interesting to note that there is a high concentration in tumor tissue and especially in osteomyelitic bone.

The next two cases were treated with PMT intravenously. In a 56-year-old man with a large pheochromocytoma, which was removed at operation, there

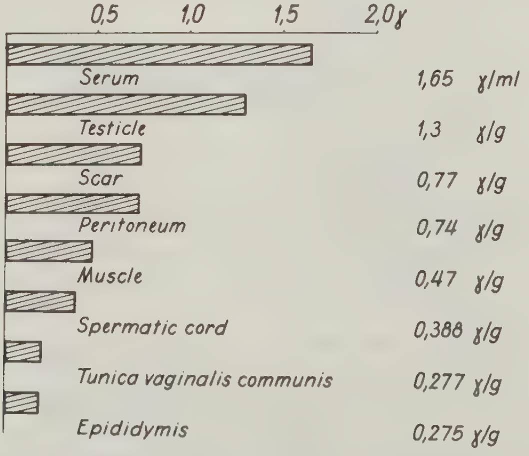

Fig. 4. Serum and tissue concentrations of PMT (250 mg/day, i.v., for 1 day) in a 48-year-old male with recurrent inguinal hernia who had undergone a herniotomy and semicastration.

were relatively high levels in the wall of the tumor and in the tumor itself. These findings are shown in Fig. 3.

The last example serves to demonstrate the high concentrations attained under pathological conditions in chronic inflammation. In this case of recurrent hernia, high concentrations were found in the testicle and in the scarred hernia sack. The data are presented in Fig. 4.

These investigations, which are still going on, have so far yielded the following facts:

The former opinion that there is a direct relation between tissue and serum levels was not borne out by our findings. The serum level by itself does not give a true estimate of the presumable level in various tissues. Therefore it does not allow a definitive statement about the therapeutic effect of an antibiotic substance. High concentrations of DMCT and PMT were found in tumor tissue and in tissues undergoing inflammatory changes, namely in the gall bladder, gall fluid, lung, bone, nerves, appendix, testicle, ovary, fallopian tube, cervix, and vaginal mucosa. One cannot yet answer the question of whether or not there is such a thing as actual accumulation of antibiotic substance in tissue.

DISCUSSION

DR. ANDERSON: I'm going to stick my neck out here. Aren't the differences in serum and tissue levels a reflection of the vascularity? You are taking it directly from an intravenous specimen and you are taking it from the surface of the skin, so the concentration of the drug is going to be definitely less. Would the doctor please reply to that? I have one other question; if you had taken heart valve would you have found any there where you have a relatively nonvascular tissue? Wher we treat subacute bacterial endocarditis by antibiotics the drug is in the serum and then absorbed in the tissue. Is that true or untrue?

TRANSLATOR: Dr. Kuemmerle has asked that I reply for him and I shall try to do this as well as I can. He says the first case he demonstrated was a chronic empyema and there was no major vascularization in this tissue. Vascularization in subacute bacterial endocarditis depends mainly on the stage. In acute stages you would expect more vascularization than in chronic stages. He is of the opinion that vascularization is not as important as the chemical structures and chemical properties of the substance in question.

DR. ANDERSON: I was asking whether the doctor thinks the decreased levels that you got in the tissues were a reflection of the difference in the vascularity. That is, if there isn't much circulation through the tissues, then you don't get much drug there, or is the concentration a function of the molecular size or the structure of the drug that you are testing?

TRANSLATOR: Dr. Kuemmerle said, "At this time I can't give a definitive answer to your question, but I'm sure, that the serum level is not the main cause for the concentration of drugs in tissue."

FURTHER STUDIES ON FLUORESCENCE OF TETRACYCLINE ANALOGUES IN PATHOLOGICALLY CHANGED TISSUES

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The best method of studying the problem of the absorption of tetracycline analogues by pathologically changed tissue is by macroscopic or microscopic observation of their distribution with the help of fluorescence under ultraviolet light [1-9]. New findings which hitherto could not be established by any other procedure, such as the absorption into bone tissue in physiological or pathological conditions, absorption into tumorous tissue [10], and absorption into fatty necroses in acute pancreatitis [6], could be furnished by this fluorescence method.

This paper is the report of a continuation of former studies dealing with the distribution of tetracycline analogues in the organism by means of the fluorescence method. In our former study we have concentrated mainly on the question of tetracycline analogue absorption into the tissue during various pathological changes, such as specific and nonspecific inflammations, healing of wounds, healing of bone fractures, necrotic processes, and tumorous diseases. In this study we have gone further and have approached the problem from the angle of tetracycline analogue absorption into individual organs under pathological conditions. We have found that organs can be classified into three groups according to the method of tetracycline analogue absorption. Group 1 includes organs in which tetracycline analogue concentration is similar to or only slightly higher than that of the blood. Group 2 includes organs with an accumulation of tetracycline analogues. These are mainly the organs (kidneys, liver) which excrete tetracycline analogues from the organism and from the bone tissue. Organs such as the brain and the pancreas which absorb tetracycline analogues only slightly or not at all comprise group 3.

Pathological conditions substantially change this scheme. An investigation of the first group shows that the absorption and fixation of tetracycline analogues change only slightly in sterile inflammations such as might be found in the intestinal walls. On the other hand, a slight irritation of other tissues is enough

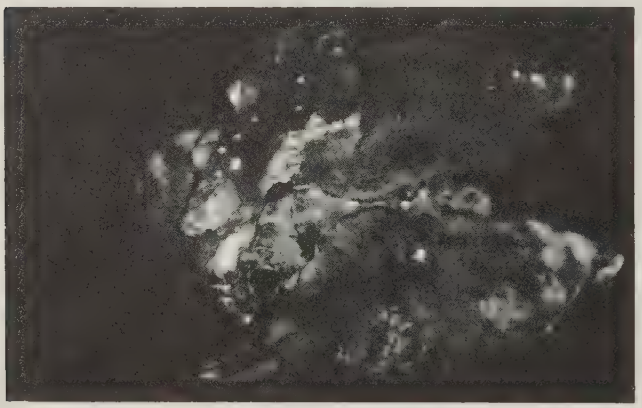

Fig. 1. Fluorescence of bronchopneumonia foci in rabbit lung 3 hr after intravenous administration of chlortetracycline.

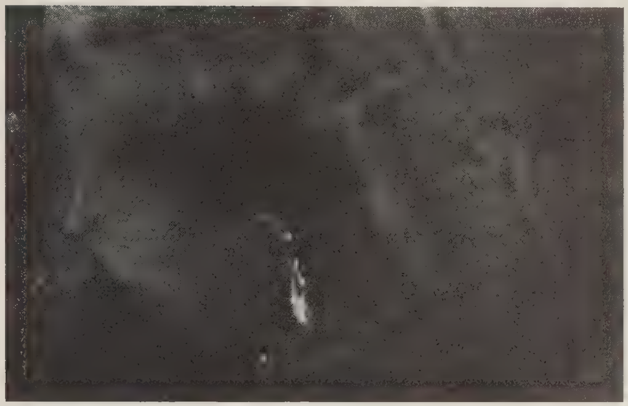

Fig. 2. Fluorescing periphery of an experimentally produced gastric ulcer in a dog 6 hr after administration of chlortetracycline.

to produce a rapid and intensive fluorescence in the damaged area. In such cases, tetracyclines also persist for a long time and after a single administration fluorescence is, as a rule, still present after 24 and even 48 hr. This is the case, for example, with lung, muscle, and skin. An illustration of such findings is given in Fig. 1 showing the lung tissue where an intensive fluorescence can still be seen in the foci of bronchopneumonia 24 hr after intravenous administration of chlortetracycline. We also could corroborate the findings of Hakkinen regarding the concentration of tetracycline analogues in the periphery of an experimentally produced gastric ulcer (Fig. 2). It is also interesting to note that tetracycline analogues penetrate heavily into arteriosclerotic vessels in the most severely damaged site. Yet the absorption is not so intensive in necrosis of the pancreas and the fixation of tetracycline analogues in arteriosclerotic plaques is also of short duration.

Concerning the second group of organs, we should like to point out the particular distribution of individual tetracycline analogues in the kidney tissue of a rabbit. An interesting feature is the varied distribution of tetracycline analogues in the kidney tissue. There is a very intensive chlortetracycline fluorescence in the cortex but considerably less in the medulla (Fig. 3). With oxytetracycline the ratio is reversed, the cortex showing a relatively slight fluorescence, whereas it is intensive in the medulla (Fig. 4). Demethylchlortetracycline behaves like chlortetracycline. As far as tetracycline distribution is concerned, it lies somewhere between chlortetracycline and oxytetracycline, and the cortex as well as

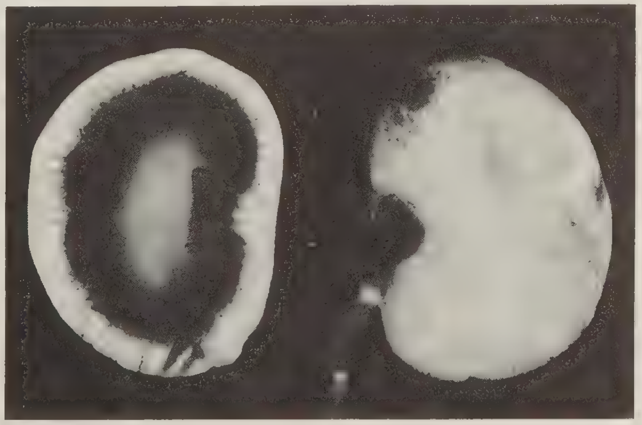

Fig. 3. Typical picture in the kidney after chlortetracycline administration. Fluorescence is most intensive in the cortex.

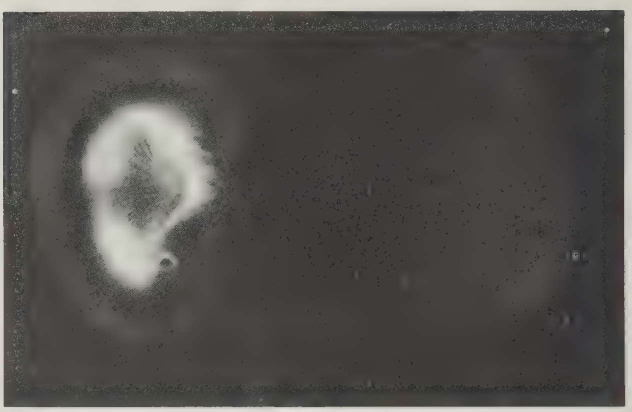

Fig. 4. Typical picture in the kidney after oxytetracycline administration. Fluorescence is most intensive in the medulla.

the medulla show a relatively even spread of fluorescence. Fluorescence in the glomerules is microscopically negligible, being manifest only in the cells of the tubules and far more intensively with chlortetracycline than with oxytetracycline. We could, therefore, corroborate the findings of Andre [11] and Bottiger [1-3] inasmuch as we were able to prove the presence of tetracycline analogues in the cells of the kidney tubules. Our contribution, however, consists in establishing the interesting fact of the varied distribution of individual tetracycline analogues in the kidney tissue.

The distribution of individual tetracycline analogues in kidney tissue is changed in pathological conditions. The fluorescence of chlortetracycline in the cortex is reduced under shock conditions. Irregular foci of fluorescence are formed under hypoxemia. In experimental staphylococcal infection of the kidney, fluorescence is intensive mainly in the area around the abscess. In experimental pyelonephritis the distribution of the individual tetracycline analogues takes on different forms. There is a focal fluorescence in the tissue with comparatively preserved function. However, it is interesting that fluorescence increases in the cells of the tubules and at the site of hyaline droplet formation (Fig. 5). In hydronephrosis, fluorescence often fades irregularly in proportion to the necrosis of the parenchyma.

In this paper we have given only a brief account of some new findings on the distribution of tetracycline analogues in pathologically changed tissues. A more detailed analysis of the individual organs will be given in subsequent papers.

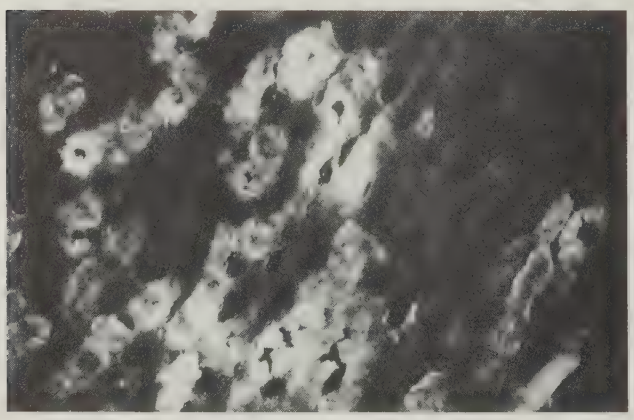

Fig. 5. Microphotograph of the kidney cortex in experimentally produced pyelonephritis after chlortetracycline administration.

The study of tetracycline analogue absorption by pathologically changed tissue is very important from the practical point of view, since it enables us to administer antibiotics under pathological conditions more purposefully and rationally.

SUMMARY

Some further findings on the absorption of tetracycline analogues by tissues under pathological conditions are reported on the basis of experimental studies. We have presented examples of absorption by foci of bronchopneumonia in the lungs, by the periphery of a gastric ulcer, and by arteriosclerotic plaques. We have proven the different distribution of chlortetracycline and oxytetracycline in kidney tissue and have shown that chlortetracycline fluoresces more intensively in the cortex, whereas oxytetracycline does so in the medulla. Finally, we reported changes of distribution of tetracycline analogues in staphylococcal infections of the kidney, in experimental pyelonephritis, and in hydronephrosis. The practical significance of these findings has been stressed.

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THE CHAOTIC STATE OF PENICILLIN SENSITIVITY TESTING AS IT EXISTS IN THE UNITED STATES

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INTRODUCTION

Every clinician, pathologist, bacteriologist, medical technologist, and manufacturer of antibiotic discs and antibiotics must become aware that penicillin sensitivity testing as practiced in the clinical bacteriology laboratories in the United States is chaotic.

We first became aware of this serious situation in 1959 [1], when we surveyed sensitivity testing procedures that were in use in 54 of the leading hospitals in Boston, New York, Philadelphia, Pittsburgh, Washington, St. Louis, and Chicago.

This chaotic state is primarily associated with the use of the dry-disc method of antibiotic sensitivity testing, which was the method used in 50 of the 54 hospitals surveyed. Since the development of the disc test by Bondi et al. [2], numerous modifications have been made. Each change moved in a different direction, with the result that today many diverse methods and interpretations are used for penicillin sensitivity testing by the disc method. These techniques and methods of interpretation vary so widely that not only is agreement of results between laboratories often impossible, but repeat tests on a single culture may vary from day to day.

Two of the modifications affecting this test are the number of discs and the amount of antibiotic to be used on each disc. For example, one hospital used three discs which contained 2, 5, and 10 units of penicillin; six hospitals used two discs containing 2 and 10 units; 20 hospitals used single discs containing

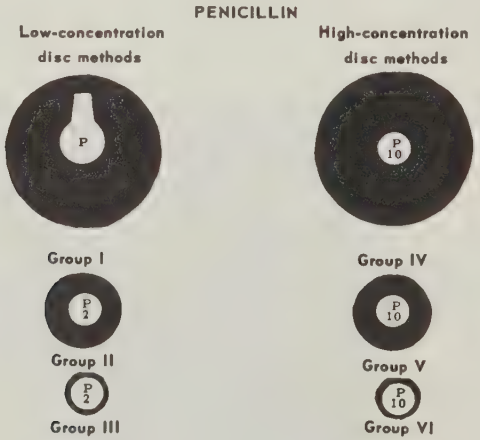

Fig. 1. Various zone size requirements used by different investigators to determine whether an organism is sensitive or resistant to penicillin.

Table 1. Penicillin Sensitivity: Interpretations of Different Methods

Culture	Zone size, mm		Group					Organism
	2-unit disc	10-unit disc	II	III	IV	V	VI	
1	27	32	S	S	S	S	S	*Staphylococcus aureus* NPP
2	28	33	S	S	S	S	S	*Staphylococcus aureus* NPP
3	30	35.5	S	S	S	S	S	*Staphylococcus aureus* NPP
4	29	36	S	S	S	S	S	*Staphylococcus aureus* NPP
5	25	31	S	S	S	S	S	*Staphylococcus aureus* NPP
6	22	31	S	S	S	S	S	*Staphylococcus aureus* NPP
7	28	38	S	S	S	S	S	*Staphylococcus aureus* NPP
8	27.5	33	S	S	S	S	S	*Staphylococcus aureus* NPP
9	30	36	S	S	S	S	S	*Staphylococcus aureus* NPP
10	30	38	S	S	S	S	S	*Staphylococcus aureus* NPP
11	30	33.5	S	S	S	S	S	*Staphylococcus aureus* PP
12	18	18	S	S	R	S	S	*Staphylococcus aureus* PP
13	13	17.5	MR	S	R	S	S	*Staphylococcus aureus* PP
14	12	15	MR	S	R	S	S	*Staphylococcus aureus* PP
15	11	13.5	R	S	R	MR	S	*Staphylococcus aureus* PP
16	11	14	R	S	R	S	S	*Staphylococcus aureus* PP
17	10.5	15	R	S	R	S	S	*Staphylococcus aureus* PP
18	10.5	16	R	S	R	S	S	*Staphylococcus aureus* PP
19	10	12	R	S	R	MR	S	*Staphylococcus aureus* PP
20	10	13.5	R	S	R	MR	S	*Staphylococcus aureus* PP
21	–	16	R	S	R	S	S	*Staphylococcus aureus* PP
22	–	13	R	R	R	MR	S	*Staphylococcus aureus* PP
23	–	125	R	R	R	MR	S	*Staphylococcus aureus* PP
24	–	13	R	R	R	MR	S	*Staphylococcus aureus* PP
25	–	14	R	R	R	MR	S	*Staphylococcus aureus* PP
26	–	13.5	R	R	R	MR	S	*Staphylococcus aureus* PP
27	–	14	R	R	R	MR	S	*Staphylococcus aureus* PP
28	–	125	R	R	R	MR	S	*Staphylococcus aureus* PP
29	–	11	R	R	R	R	S	*Staphylococcus aureus* PP
30	–	–	R	R	R	R	R	*Staphylococcus aureus* PP
31	–	–	R	R	R	R	R	*Staphylococcus aureus* PP
32	–	–	R	R	R	R	R	*Staphylococcus aureus* PP
33	–	–	R	R	R	R	R	*Staphylococcus aureus* PP
34	–	–	R	R	R	R	R	*Staphylococcus aureus* PP
35	11	13	R	S	R	MR	S	*Salmonella newport*
36	11	16	R	S	R	S	S	*Salmonella infantis*
37	–	10	R	R	R	R	S	*Salmonella montevideo*
38	–	16	R	R	R	S	S	*Salmonella oranienberg*
39	14	16	MR	S	R	S	S	*Salmonella schottmuelleri*
40	15	17	MR	S	R	S	S	*Salmonella typhosa*
41	16	19	S	S	R	S	S	*Salmonella typhosa*
42	17	21	S	S	R	S	S	*Salmonella typhosa*
43	17	20	S	S	R	S	S	*Salmonella typhosa*
44	17	19	S	S	R	S	S	*Salmonella typhosa*
45	12	14.5	MR	S	R	MR	S	*Proteus mirabilis*
46	13	18	MR	S	R	S	S	*Proteus vulgaris*
47	10	15	R	S	R	S	S	*Proteus morgani*
48	12	19	MR	S	R	MR	S	*Proteus mirabilis*
49	–	10.5	R	R	R	R	S	*Proteus rettgeri*
50	–	–	R	R	R	R	R	*Proteus rettgeri*

S = sensitive, R = resistant, MR = moderately resistant, NPP = non-penicillinase producer, PP = penicillinase producer.

2 units; 18 hospitals used a single disc containing 10 units; and five hospitals used single discs containing 5 units.

Other modifications which affect the results are the different criteria for determining what zone size around a disc should identify an organism as sensitive or resistant. Figure 1 shows that the advocates of the 2-unit disc for penicillin are divided into three groups. Group I defines as sensitive any organism which produces a large zone of inhibition around the disc (15 mm from the edge of the disc to growth, using a "Consolidated" disc). Group II states that an organism is sensitive if there is a medium-size zone or greater present (15 mm total diameter). Group III considers an organism sensitive it there is any distinct zone of inhibition around the disc. The advocates of the 10-unit disc are divided along similar lines, as indicated by Groups IV, V, and VI. Somewhat similar subdivisions occur with the 5-unit discs. Most investigators consider organisms which do not fit the above classification to be either moderately resistant or resistant, depending upon specified criteria.

To determine experimentally the effect of the methods of interpretation on results of penicillin sensitivity testing, we selected 50 cultures of pathogens varying in resistance from very sensitive through very resistant. These cultures were tested by Mr. Kirschbaum and Mr. Kramer of the Antibiotic Division of the Food and Drug Administration with 2- and 10-unit penicillin discs. The zone sizes are given in Table 1. To demonstrate that the diverse methods of interpretation produce a host of conflicting results, we simply apply the different methods of interpretation illustrated in Fig. 1 to each of the 50 cultures. The method used by Group I could not be used because a different style disc is used by these laboratories. The divergent results obtained are shown in Table 1.

Another modification affecting this test is the use by many laboratories of primary cultures with uncontrolled inoculum sizes. To demonstrate the variable effect of primary cultures on the results obtained in clinical laboratories a single culture of a penicillinase-producing Staphylococcus aureus was seeded on a large trypticase soy agar plate, using various size inocula such as are encountered in clinical specimens. Each inoculum was tested with two- and ten-unit penicillin discs. The plates were incubated overnight. Table 2 lists the zone sizes and the divergent set of results obtained when these zone sizes are interpreted by the methods of Groups II, III, IV, V, and VI.

Another factor affecting the result is the use of defective discs. In a small survey during the past six months we found that several laboratories were using

Table 2. Effect of Varied Inoculum Size on Penicillin Sensitivity Test Results

Specimen	Zone size, mm		Interpretation				
	2-unit disc	10-unit disc	II	III	IV	V	VI
1	–	–	R	R	R	R	R
2	–	9	R	R	R	R	S
3	9	11	R	S	R	R	S
4	12	14	MR	S	R	MR	S
5	15	19	S	S	R	S	S
6	16	20	S	S	R	S	S
7	16	21	S	S	R	S	S

penicillin discs that were outdated from three to six months. The results on a selected set of cultures deviated so markedly from the expected results that we tested the discs for penicillin performance. The 10-unit discs performed like 1-unit discs and the 2-unit discs like 0.5-unit discs. These were the operational discs used in clinical laboratories! Many cultures tested by the methods in use by these laboratories should have been called sensitive but were reported as resistant. Defective discs today err in the direction of being underloaded. Erroneous results from defective discs err in the direction of calling bacteria resistant when they should be reported as sensitive.

DISCUSSION

The selection of an antibiotic in hospitals for the treatment of infections, which at times are life-threatening, is made on the basis of sensitivity tests performed on cultures isolated from the patient. The foregoing results have shown that three variables, (a) method of interpretation, (b) inoculum size, and (c) defective discs that may interplay and produce a spectrum of divergent results. The results vary so widely that agreement of results from laboratory to laboratory is often impossible and results on a single culture when repeated from time to time change from day to day. Which method of interpretation is right is in dispute. But we may conclude that if the method used by Group IV is correct (which identified 11 of the 50 cultures as sensitive) then the others are wrong. If this is the case, then penicillin is being given to patients uselessly. On the other hand, if the method used by Group VI is right then patients in the other institutions are being denied antibiotics which could be effective. Similar deductions can be made for the other methods.

In the early years following the introduction of penicillin this confusion was understandable. However, after 15 years such a situation is intolerable. Frequently the question is asked, "What can be done about it?" It has been suggested that since many of the people responsible for these conditions are present at this meeting the doors be locked and no one allowed to leave until judicious conclusions have been reached. In our opinion this would serve no useful purpose. After 15 years of penicillin testing each sponsor of a procedure has developed strong convictions about his method and righteously defends his position. It would be like getting a Catholic priest, a Jewish rabbi, a Methodist minister, and a Moslem imam in the same room and having Billy Graham try to convert each to the Baptist faith.

Since this test has been devised to serve, it is now time that the views of all the proponents were obtained, the results evaluated, and a standard procedure developed to which clinicians, pathologists, bacteriologists, and manufacturers of antibiotic discs and antibiotics can agree. If this can be accomplished then physicians in Dallas, San Francisco, and New York will all be given comparable reports for similar organisms instead of the often misleading, unsatisfactory results being reported today.

A step in this direction has been taken by the World Health Organization. Data in our possession regarding the ultimate solution of this problem are in keeping with these findings. In order to hasten the day when uniform sensitivity tests may be achieved we suggest that the Council on Microbiology of the American Society of Clinical Pathologists review the problem as sitting judges and review the accumulated data in the light of the World Health Organization report. If the committee feels that current procedures of sensitivity testing are unsatisfactory and need revision, the Council could recommend a standard method. In the interest of better patient care we would hope that the procedure would meet with national acceptance and be adopted by laboratories, manufacturers of antibiotics and discs, and governmental agencies.

CONCLUSION

A survey showed marked disagreements in methods of interpretation and methods of performing the penicillin disc tests. These methods and techniques, when applied to a set of 50 cultures of varying sensitivities, resulted in widely divergent patterns of sensitivity. It is suggested that a responsible organization accumulate data on this subject and recommend uniform procedures. It is our hope that all people will soon realize that at the present time, of all the variables to be considered, the most stable in these tests is the bacterium, and the most unstable the group of people studying it.

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DISCUSSION

DR. SCHERR (Consolidated Laboratories, Chicago): I think the data you have presented are excellent in showing some of these discrepancies. Now, I think I might reject categorically the conclusion that any one method of determining what is sensitive is correct and another one is incorrect, because Kirby's method, for example, works perfectly if you follow Dr. Kirby's directions for interpreting and performing the tests. So in the hands of a single laboratory, using Kirby's method and his conclusions, the test works fine and you will get proper results. And in another laboratory down the street, using somebody else's method and interpretation, it will work fine for him. And both of these investigators in separate laboratories should end up with the same ultimate answer, namely, what is the best antibiotic to use for the patient. The question is not which one method is correct. The question is: can a standard method be developed which may not be the best one in the world, but which would be used uniformly throughout the United States, if not the world? Now, such an attempt was made by the Food and Drug Administration in the United States by calling together, over a period of about two years, experts in the field, at which session Mr. Fowler himself was present, as well as manufacturers of discs and representatives of various agencies concerned here. And they did agree on a quasi standard method, which, apparently, you now reject, pointing to it as a poor method for determining sensitivity testing. I deny that this is so, since any standard method, no matter how poor it is, is excellent as long as everybody uses it and the interpretations are consistent from one laboratory to the other.

DR. FOWLER: I can answer this way. I don't agree that the results are uniform from hospital to hospital. In our experience that doesn't occur.

DR. BRANCH (Canada): Dr. W. W. Wright and I were honored by being asked to attend the meeting on antibiotic sensitivity testing in Geneva in July. The report only came to hand this week and as I understand it, there is going to be some effort to discuss these findings this evening.

CLINICAL AND SERUM-LEVEL STUDIES WITH AN INTRAMUSCULAR PREPARATION OF OXYTETRACYCLINE*

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OBJECT OF INVESTIGATION

The investigation to be described was originally stimulated by experience with large doses of parenteral oxytetracycline in two seriously ill children. These children, both of whom were under the age of five, were admitted to the hospital with severe generalized peritonitis following perforation of a gangrenous appendix. In addition, one of the children was suffering from a right-sided pneumonia. At that time the manufacturers' recommended dosage for intravenous oxytetracycline was 100 mg q.i.d. However, these children were both so seriously ill that each was given 500 mg of oxytetracycline q.i.d. in a glucose-saline drip. Within 36-48 hr their condition was dramatically improved.

Shortly afterwards a parenteral preparation of oxytetracycline which was preconstituted and suitable for intramuscular injection became available. The experience described above stimulated a series of investigations into various regimes of administration of this intramuscular preparation with a view to assessing, in terms of serum antibiotic levels, the minimum frequency of injection which would ensure continuous therapeutic serum levels of the antibiotic. For the purpose of this investigation the minimum therapeutic serum level was taken to be 0.6 μg/ml of serum.

MATERIAL

The preparation used was a new preconstituted intramuscular solution of oxytetracycline described originally by Hammer et al. [1]. This consisted of a solution of an ethanolammonium magnesium salt of oxytetracycline. Laboratory investigations have demonstrated that solution of this antibiotic salt results in a convenient, stable preparation, which is well tolerated by intramuscular injection. The formulation is completed by the addition of a suitable antioxidant and 2% lidocaine.†

The oxytetracycline solution was preconstituted and yellow to light amber in color, according to the concentration. No refrigeration is required and color and potency are stable under normal conditions of storage, provided long exposure to air is avoided. Stability and accelerated-stability tests at 37 and 50 C indicate that the potency is satisfactorily retained for approximately 24 months at room temperature.

The concentration of oxytetracycline used in the investigations described was 125 mg/ml and 50 mg/ml. The former concentration was used exclusively except in regimes which required single injections of 100 mg.

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*The trade name of Pfizer Limited for oxytetracycline is Terramycin.

†The trade name of Astra Pharmaceutical Products for lidocaine is Xylocaine.

INJECTION TECHNIQUE

The site of injection selected in this study was the upper third of the lateral aspect of the thigh, as recommended by Grey Turner. The more usual site of the upper outer quadrant of the buttock was not employed because of the pain or discomfort and local intolerance which may occur with intramuscular injections in obese individuals in whom the site of deposition of the injected material may actually be superficial to the gluteal muscles. Furthermore, in such obese individuals the gluteal muscles may be heavily infiltrated with fat and this may result in local intolerance to such a preparation. On several occasions the author has had to open abscesses in the buttock resulting from intramuscular injections in the upper outer quadrant. These difficulties may be obviated if the upper third of the lateral aspect of the thigh is used and care is taken that the needle is inserted perpendicular to the skin surface.

CASE MATERIAL AND METHOD

A total of 95 patients received multiple injections of preconstituted oxytetracycline solution. Twenty of these patients were ambulant and were selected from patients presenting at the outpatient department with mild or moderate local infections for which broad-spectrum antibiotics were considered to be indicated. The remaining 75 patients had been admitted to the hospital with a variety of acute surgical conditions in which broad-spectrum antibiotics were considered to be indicated pre- or postoperatively for prophylactic or therapeutic reasons. Local tolerance to the injections was noted, together with any untoward reactions. Clinical response was also assessed.

Six regimes of administration of oxytetracycline were investigated and these are described below. During each regime, blood samples were withdrawn at times which were selected to indicate maximum and minimum levels of antibiotic in the serum. Possible cumulation of serum levels was also investigated. The regimes of administration were briefly as follows: 1) 250 mg of oxytetracycline was administered at 6-hr intervals to 13 patients; 2) 250 mg of oxytetracycline was administered at 8-hr intervals to 10 patients; 3) 250 mg of oxytetracycline was administered at 12-hr intervals to 12 patients; 4) 250 mg of oxytetracycline was administered at 24-hr intervals to 20 patients; 5) 250 mg of oxytetracycline was administered to 20 patients once daily on alternate days, i.e., days 1, 3, 5, and 7; 6) Intramuscular oxytetracycline was administered to 20 patients once daily at intervals of 24 hr. On the first day a single injection of 250 mg was given and on the five or six subsequent days a single injection of 100 mg was given. In regimes 1-4, therapy with intramuscular oxytetracycline was continued for the number of days required by the patients' clinical response.

All serum samples were assayed by a modification of the technique described by Brownlee et al. [2].

RESULTS

The results of the individual assays on serum samples are set out in Tables 1-6.

In regime 1 (Table 1), 250 mg of oxytetracycline was administered intramuscularly every 6 hr. Samples of blood were withdrawn 1, 6, 24, and 25 hr after the first injection. It is clear from these successive samples that the

Table 1. Blood Level of Oxytetracycline* Following Intramuscular Administration of 250 mg Every 6 hr

Patient No.	Sample 1, 1 hr after first injection	Sample 2, 6 hr after first injection	Sample 3, 24 hr after first injection	Sample 4, 25 hr after first injection
1	1.14	1.97	5.30	6.00
2	1.50	2.87	8.60	10.01
3	1.26	1.73	3.90	3.70
4	0.36	0.90	2.25	3.10
5	1.15	2.33	4.60	5.60
6	0.63	1.07	4.10	4.10
7	0.36	1.00	3.60	3.80
8	1.13	2.24	5.90	5.20
9	N.D.†	1.30	3.04	3.00
10	0.45	0.96	4.20	5.10
11	0.21	0.57	2.28	2.45
12	1.35	0.93	1.73	3.00
13	3.10	2.40	3.30	3.90
Mean	0.97	1.56	4.06	4.54

*Results expressed as μg/ml of serum.
†N.D. = Nil detected.

serum level of oxytetracycline showed rapid cumulation and was well maintained above the therapeutic serum level from 1 hr after the first injection onwards.

In regime 2 (Table 2), 250 mg of oxytetracycline was administered intramuscularly every 8 hr. Samples of blood were withdrawn 1, 8, 24, and 25 hr after the first injection. In this case the sample 1 hr after the first injection had not quite reached the therapeutic serum level but this level was apparently reached at some time between the administration of the first and second injections. From the time of the second injection onwards there was considerable cumulation to levels well above the minimum therapeutic serum level.

Table 2. Blood Level of Oxytetracycline* Following Intramuscular Administration of 250 mg Every 8 hr

Patient No.	Sample 1, 1 hr after first injection	Sample 2, 8 hr after first injection	Sample 3, 24 hr after first injection	Sample 4, 25 hr after first injection
1	0.62	0.69	1.95	1.92
2	0.19	0.87	2.58	3.32
3	0.70	1.06	2.65	1.88
4	0.29	0.63	1.08	1.46
5	0.53	0.88	1.72	2.55
6	0.91	0.57	1.22	1.87
7	<0.1	0.44	1.16	2.46
8	<0.1	0.44	2.43	2.08
9	1.20	0.51	2.36	2.70
10	0.20	0.36	0.57	1.13
Mean	0.46	0.65	1.77	2.14

*Results expressed as μg/ml of serum.

Table 3. Blood Level of Oxytetracycline* Following Intramuscular Administration of 250 mg Every 12 hr

Patient No.	Sample 1, 1 hr after first injection	Sample 2, 12 hr after first injection	Sample 3, 24 hr after first injection	Sample 4, 25 hr after first injection
1	0.36	0.93	2.30	2.70
2	0.27	0.45	0.81	0.96
3	0.36	0.66	1.20	1.71
4	0.42	0.36	0.81	0.81
5	0.42	0.57	0.96	1.11
6	0.27	1.22	1.95	2.40
7	1.46	1.11	2.27	3.19
8	0.95	1.37	1.96	2.01
9	1.25	1.11	0.81	1.70
10	0.78	1.40	2.00	3.00
11	1.17	1.82	2.35	3.00
12	2.40	0.93	1.58	1.71
Mean	0.84	0.99	1.58	2.03

*Results expressed as μg/ml of serum.

Table 4. Blood Level of Oxytetracycline* Following Intramuscular Administration of 250 mg Every 24 hr

Patient No.†	Sample 1, 1 hr after first injection	Sample 2, 24 hr after first injection	Sample 3, 25 hr after first injection	Sample 4, 48 hr after first injection
1	0.63	0.33	0.45	1.32
2	1.01	0.76	2.15	1.04
3	1.18	0.66	1.90	0.99
4	1.19	0.89	1.54	1.14
5	0.82	0.51	1.45	0.60
6	0.94	0.42	1.40	0.55
7	0.62	0.80	2.05	1.42
8	2.74	1.02	2.07	0.70
9	1.44	0.84	2.40	0.84
10	0.93	0.71	1.56	0.81
11	0.53	0.75	1.40	0.81
12	1.20	0.72	1.47	0.86
13	0.52	0.45	0.78	0.48
14	1.02	0.27	0.67	0.45
15	1.14	0.78	2.16	0.84
16	1.26	0.66	1.44	0.96
17	1.56	0.48	2.10	0.75
18	0.57	0.39	1.00	0.48
19	1.02	0.48	1.14	0.51
20	0.83	0.81	2.40	0.90
Mean	1.06	0.64	1.58	0.82

*Results expressed as μg/ml of serum.
†Outpatients.

Table 5. Blood Level of Oxytetracycline* Following Administration of 250 mg on Alternate Days§

Patient No.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7
	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr
1	2.17	1.41	1.07	0.68	2.30	1.26	1.17	0.51	N.S.‡	1.47	N.S.	0.36	2.63
2	2.60	1.17	1.02	0.42	3.07	1.38	1.08	0.36	1.70	0.90	1.05	0.39	1.92
3	1.11	0.89	0.68	0.28	1.42	0.72	0.67	0.25	2.02	0.95	0.86	0.32	0.96
4	2.17	1.38	1.32	0.90	3.10	1.67	1.53	1.02	3.24	1.84	1.46	0.65	3.05
5	1.92	1.23	1.12	0.56	2.44	1.11	0.99	0.35	2.43	1.26	1.11	0.83	1.85
6	1.58	0.66	0.81	0.81	2.48	0.87	0.60	0.34	1.82	0.89	0.66	N.D.	2.28
7	1.80	0.57	0.51	N.D.†	0.96	0.57	0.36	0.23	2.40	0.78	0.51	N.D.	1.52
8	2.27	1.62	1.32	0.71	4.10	Broken	1.83	0.70	2.50	1.46	1.23	0.54	0.74
9	1.33	0.48	0.38	0.69	2.40	0.56	0.34	N.D.	1.23	0.62	0.51	N.D.	1.76
10	0.87	0.35	0.28	N.D.	2.50	0.60	0.50	0.17	1.30	N.S.	0.95	0.53	2.55
11	1.49	0.60	0.48	0.40	1.31	0.54	0.43	N.D.	2.00	0.66	0.51	N.D.	1.58
12	1.25	0.68	0.62	N.D.	2.57	1.09	1.08	0.52	2.97	1.21	1.38	0.47	1.91
13	1.26	2.07	1.89	0.75	0.69	0.84	0.35	N.D.	4.28	1.39	1.22	0.50	3.77
14	1.79	0.68	0.66	N.D.	1.40	0.51	0.39	N.D.	1.19	0.45	0.32	N.D.	2.13
15	2.00	1.50	1.20	0.50	3.60	1.80	1.60	0.60	5.00	2.30	1.40	0.73	4.20
16	1.80	1.20	0.65	1.70	1.70	1.10	1.00	0.30	1.60	1.30	1.10	0.50	3.25
17	1.80	1.50	2.10	1.10	1.70	0.90	0.60	N.D.	3.70	1.30	1.00	N.D.	2.40
18	1.80	1.10	0.80	0.50	2.20	1.40	1.20	1.10	2.20	1.10	0.80	N.D.	2.80
19	2.51	1.21	1.11	0.59	1.78	1.01	1.01	0.36	2.92	1.20	1.33	N.D.	2.45
20	1.80	1.87	1.92	1.68	3.49	2.19	1.65	1.53	3.94	2.04	1.62	0.59	2.82
Mean	1.77	1.11	0.99	0.61	2.26	1.06	0.92	0.42	2.55	1.22	1.02	0.32	2.33

*Results expressed as μg/ml of serum.
†N.D = Nil detected.
‡N.S = No sample.
§Injections were given on days 1, 3, 5, and 7.

Table 6. Blood Levels of Oxytetracycline* Following Intramuscular Administration of 250 mg on Day 1 and 100 mg Daily Thereafter

Patient No.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7
	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr
1	1.25	0.72	1.58	0.63	1.80	0.45	1.43	0.33	1.69	0.48	1.34	0.28	1.33
2	2.90	0.78	1.80	0.63	2.04	0.39	0.93	0.30	1.02	N.D.	0.90	N.D.	1.33
3	1.92	0.81	1.43	0.39	1.47	0.20	1.32	0.28	1.80	0.29	1.46	0.23	2.20
4	1.62	1.03	2.64	1.19	2.01	1.17	2.40	0.70	2.36	1.02	1.98	0.39	2.04
5	2.30	1.80	2.90	1.50	2.20	0.63	2.40	0.60	1.68	0.36	2.28	N.D.	2.00
6	1.32	0.93	1.50	0.48	1.77	0.57	1.65	N.D.	1.11	0.60	1.41	N.D.	1.62
7	1.94	1.60	3.60	1.78	4.23	2.08	3.56	1.65	3.12	1.48	2.85	1.49	2.98
8	1.67	0.99	1.48	0.62	2.27	0.64	2.03	0.58	0.33	N.D.	2.46	1.04	2.34
9	2.60	1.59	2.12	1.61	2.38	1.28	2.57	1.22	2.27	1.23	2.02	0.99	1.86
10	1.46	0.60	1.16	0.53	1.26	0.50	1.17	0.32	1.32	0.27	1.11	0.36	1.38
11	1.42	0.89	1.52	0.64	1.69	0.58	1.50	0.38	1.32	0.41	1.38	0.47	1.49
12	1.14	0.66	1.10	0.51	1.03	0.33	1.30	0.36	1.50	0.38	N.D.	N.D.	1.28
13	1.40	0.84	0.93	0.27	1.32	0.35	1.50	0.28	1.22	0.30	1.13	0.29	1.44
14	N.D.†	N.D.	1.43	1.30	3.40	2.32	4.95	2.98	3.09	2.10	4.85	2.12	4.25
15	1.48	1.20	2.01	0.93	1.60	0.60	2.88	1.29	1.96	0.80	1.77	0.40	2.04
16	1.42	0.73	1.32	0.72	1.80	0.48	1.48	0.33	1.75	0.38	1.56	0.44	1.72
17	1.26	0.84	0.78	0.41	1.34	0.48	1.61	0.50	1.38	0.65	1.94	0.50	2.35
18	2.43	0.96	1.42	0.63	1.52	0.65	1.60	0.36	1.65	0.57	1.31	0.41	1.46
19	1.60	1.60	1.50	1.20	1.70	1.20	1.80	1.40	4.00	1.60	2.10	1.00	4.10
20	1.92	1.16	2.45	0.93	2.98	0.90	1.14	0.50	1.50	0.52	2.10	N.D.	1.84
Mean	1.65	0.99	1.73	0.85	1.99	0.80	1.96	0.72	1.80	0.67	1.80	0.52	2.05

*Results expressed as μg/ml of serum.
†N.D. = Nil detected.

In regime 3 (Table 3), 250 mg of oxytetracycline was administered intramuscularly every 12 hr. Samples of blood were withdrawn 1, 12, 24, and 25 hr after the first injection. The minimum therapeutic serum level was reached by 1 hr after the first injection and cumulation again occurred although less rapidly than with regimes 1 and 2.

In regime 4 (Table 4), a single injection of 250 mg of oxytetracycline was administered intramuscularly at intervals of 24 hr. Samples of blood were withdrawn 1, 24, 25, and 48 hr after the first injection. One hour after the first injection the mean serum level was considerably in excess of the minimum required and was still maintained somewhat above the minimum in the sample taken immediately preceding the second injection. A comparison of the sample taken 1 hr after the second injection with that taken 1 hr after the first injection indicates that slight cumulation is taking place and that the minimum therapeutic serum level is just maintained from a period of 1 hr after the first injection onward.

In regime 5 (Table 5), 250 mg of oxytetracycline was administered once daily on alternate days. Two blood samples were withdrawn each day 4 and 24 hr after the time of injection. In the case of days 2, 4, and 6, on which no injection was given, samples of blood were withdrawn at times corresponding to the times of withdrawal on days 1, 3, 5, and 7. While the serum levels 4 hr after each injection showed some degree of cumulation, the levels on the intervening days fell below the figure which is considered to be satisfactory and this regime, therefore, does not provide a continuous serum level of oxytetracycline of 0.6 μg/ml or greater.

In regime 6 (Table 6), 250 mg of oxytetracycline was given as a single intramuscular injection on the first day, followed by a single injection of 100 mg oxytetracycline given intramuscularly on days 2 to 7. Samples of blood were withdrawn 4 and 24 hr after each injection. Inspection of the results shows that the initial 250-mg injection provides a very satisfactory level which subsequent daily injections of 100 mg do not quite maintain; for example, the second blood sample on the sixth day shows a level of only 0.52 μg/ml. In addition it will be seen that the levels of the second samples from the second day onwards show a gradual reduction, so that it cannot be said that this regime will provide a continuous serum level in excess of 0.6 μg/ml except during the first two or three days.

No allergic or other untoward reactions were seen in any patient in this series. In particular, there was no evidence of drug fever or of gastrointestinal disturbance attributable to the antibiotic. Local tolerance to multiple injections was considered excellent in all inpatients. In outpatients who were receiving single injections of 250 mg of oxytetracycline at 24-hr intervals, however, there were complaints of moderate local intolerance for some hours after the injection in 50% of cases. In no case was the local reaction so severe as to evoke unwillingness on the part of the patient to have subsequent injections.

In regimes 1-3 the clinical response was regarded in all cases as excellent. In regime 4 the clinical response was considered either good or excellent. However, comparison of the clinical response in patients on regimes 5 and 6 with previous experience of larger daily doses of the antibiotic indicates that these regimes result in a delayed or incomplete therapeutic response.

DISCUSSION

The formulation of intramuscular oxytetracycline used in this study overcomes some of the disadvantages associated with other presently available forms of tetracyclines for intramuscular injection. The propylene glycol formulation is stable, convenient to administer, and is well tolerated locally and generally.

In terms of a minimum therapeutic serum level of 0.6 μg/ml, administration of 250 mg of the antibiotic at 6-, 8-, or 12-hr intervals results in a rapid rise of serum levels to figures well above the specified minimum. Such regimes, therefore, may be expected to be more than adequate for the management of infections of any severity due to tetracycline-sensitive organisms and this is confirmed by the clinical responses.

In the case of the regime in which 250 mg every 24 hr is administered, the minimum therapeutic serum level appears to be just maintained during the whole of the first 24 hr but some degree of cumulation takes place during the second day. This regime, therefore, may be considered adequate for mild or moderately severe infection.

In those regimes (5 and 6) which involve the administration of less than 250 mg of oxytetracycline daily, continuous therapeutic serum levels are not provided and analysis of the clinical response indicates that in fact, these two regimes do not provide adequate therapy.

Statistical analysis of all the results indicates that in infections of any severity adequate therapy would be provided two to four injections of 250 mg each during the first 24 hr followed by a maintenance regime from the second day onwards of a single injection of 250 mg daily.

ACKNOWLEDGEMENTS

I wish to thank, in particular, Dr. J. K. Morrison, Head of Clinical Research Pfizer, Ltd., for his advice and guidance in this investigation as well as in the preparation of this paper. I would also like to thank Messrs. Millward, Herbert, and Smith for allowing me to investigate their cases and the nursing staff for their cooperation, particularly Sister M. Scott for her invaluable assistance. My thanks are also due to Mr. Weber for his excellent technical assistance.

The assay of serum samples was kindly carried out by the Microbiological Department of Pfizer, Ltd., Sandwich, England.

SUMMARY AND CONCLUSIONS

A preconstituted intramuscular preparation of oxytetracycline has been administered in accordance with six differing regimes to a total of 95 patients. Seventy-five of these patients were seriously ill inpatients. Assessment of clinical response and correlation of this response with the antibiotic serum level provided by each of the regimes indicates that satisfactory therapy is provided by regimes 1-4, which are described. For very severe infections it is recommended that two or more injections of 250 mg each be given during the first 24 hr, with a single injection of 250 mg on the second and subsequent days. The preparation is well tolerated locally and generally and no case of drug allergy or other untoward reaction were recorded. The preparation is convenient and stable for prolonged periods at room temperature and may therefore be said to overcome some of the disadvantages of earlier intramuscular preparations of tetracyclines.

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DISCUSSION

DR. MANIAR (Ottawa): I would like to ask what was the pH of your final solution and the method and the organisms used in the biological assay of serum?

DR. STILLMAN: Dr. Jackson might know what the actual pH was. I don't know the actual pH. The clinical response was the important thing. We don't think there was much as far as the organism goes. The incriminating organisms were not actually isolated. The infections were generally due to a mixture of organisms as, for example, in peritonitis. I myself don't know which test organisms the laboratory used for assay.

THE EFFECT OF MEXAFORM ON THE INTESTINAL FLORA OF RATS

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INTRODUCTION

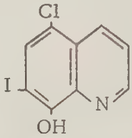
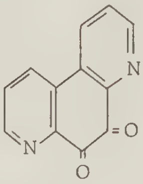
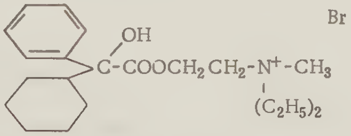
It has been established clearly that prolonged oral administration of certain antibiotics, particularly neomycin, polymyxin, and the tetracyclines, frequently results in a marked increase in the number of yeasts (Candida) in the intestinal tract and upon the oral mucous membranes. Concurrently, the normal bacterial population of the intestine, especially the coliforms, is substantially reduced [1-4]. This disturbance of the normal balance of the intestinal and oral flora frequently gives rise to moniliasis.

Attempts to prevent such an increase in yeast numbers as a result of oral antibiotic administration have led to the concurrent use of selected antifungal antibiotics. Tetracycline plus nystatin [5, 6], neomycin plus nystatin [7], neomycin plus polymyxin [8], and tetracycline plus pimaricin [9] are examples of such combinations. With the exception of the last combination, which has been evaluated in rats and mice, these antibiotic mixtures have been studied orally in limited numbers of patients and with equivocal results. There are few published clinical results extant regarding the curative properties of such anti-fungal antibiotics once an abundant yeast population has become well established in the intestinal tract. This lack of data may be due to the difficulties involved in obtaining microbiological studies from a sufficient number of patients with moniliasis. Well-controlled studies are needed in which normal ranges or variations with respect to the bacterial and yeast populations in the feces may be established as a basis for assessing therapeutic response.

However, in view of these practical difficulties in evaluating antifungal or other agents for the prevention or treatment of moniliasis in humans, rats serve admirably as preliminary test subjects. Manten and Hoogerheide [9] observed a large restriction in the growth of intestinal yeasts in rats by the antifungal antibiotic, pimaricin, during tetracycline administration. Tetracycline, administered alone, brought about a significant elevation in the numbers of fecal yeasts.

Recent clinical trials have shown that Mexaform, a new preparation consisting of a mixture of three synthetic compounds, exerts a curative effect upon various types of infectious enteritis including that associated with increased numbers of Candida albicans. The composition of Mexaform is shown in Table 1. Vioform® [10] and Entobax® [11-13], two of its ingredients, have antibacterial and antiprotozoal activity while the third component, Antrenyl®, is an antispasmodic. Kradolfer and Neipp [14], in a study of the chemotherapeutic activity of Entobax in rats, found that its oral administration resulted in an appreciable numerical increase in coliform bacteria in the feces. More recently, Sackmann and Kradolfer [15] have noted in rats receiving Mexaform or either of its two antimicrobial components that prior to an increase in intestinal bacterial counts there occurs a short period in which these counts are reduced to subnormal levels. These investigators attribute these changes in fecal counts to the

Table 1. Composition of Mexaform

Component	Composition, %	Structure
Vioform (antimicrobial)	90.09	 5-chloro-7-iodo-8-hydroxyquinoline
Entobax (antiprotozoal)	9.01	 4,7-phenanthroline-5,6-quinone
Antrenyl (antispasmodic)	0.90	

destruction of sensitive bacteria and the subsequent development of strains resistant to the action of these drugs.

Evidence in the literature indicates that a disturbance in the ecology of the enteric microflora of normal individuals often gives rise to secondary infections of the intestinal tract. Numerous investigators have proposed that the relatively few yeasts present in the normal flora of the intestinal tract are held in check by the coliforms, possibly as a consequence of a competition for nutrients. Miller [16] found that the reduction in the numbers of Bacteroides in the intestinal tract of mice given streptomycin was accompanied by a heightened susceptibility of these animals to infection with a streptomycin-resistant strain of *Salmonella enteritidis* administered via stomach tube.

In an effort to provide additional understanding of the favorable clinical reports on the effectiveness of Mexaform in the treatment of infectious enteritis of diverse etiology [17, 18] and in the light of Kradolfer and Neipp's findings [14] on fecal coliform increase, it was of interest to determine (a) the effect exerted by orally administered Mexaform upon the total aerobic bacterial and coliform flora of normal rats and (b) its effect on the yeast and coliform counts in rats pretreated with aureomycin.

METHODS AND RESULTS

Young male adult rats, randomly selected, were used in these experiments. They were individually caged and numbered. Fecal specimens were expressed in the morning from each rat by gentle pressure and promptly weighed and emulsified with saline solution to yield a 1% suspension. Serial dilutions were then prepared and aliquots plated in appropriate agar media. The nutrient media used were BBL trypticase soy agar, pH 7.3 (total aerobic bacteria); BBL desoxycholate

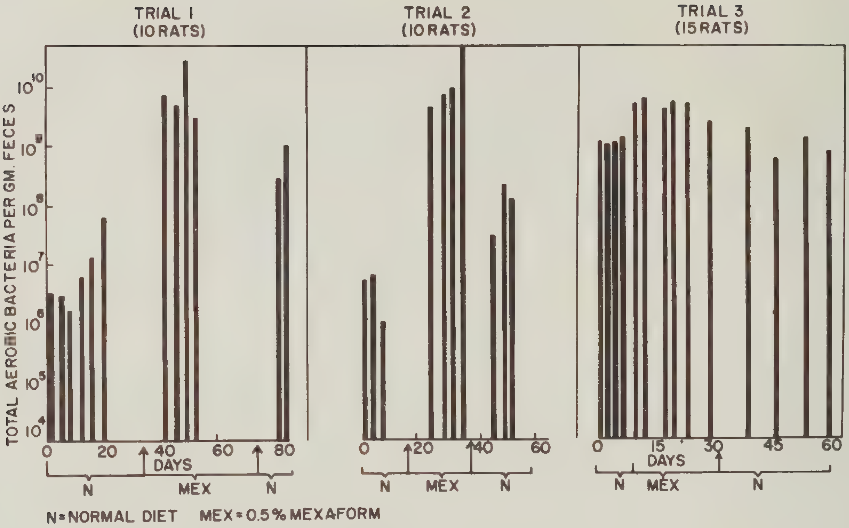


Fig. 1. Effect of Mexaform upon numbers of total aerobic bacteria in rat feces.

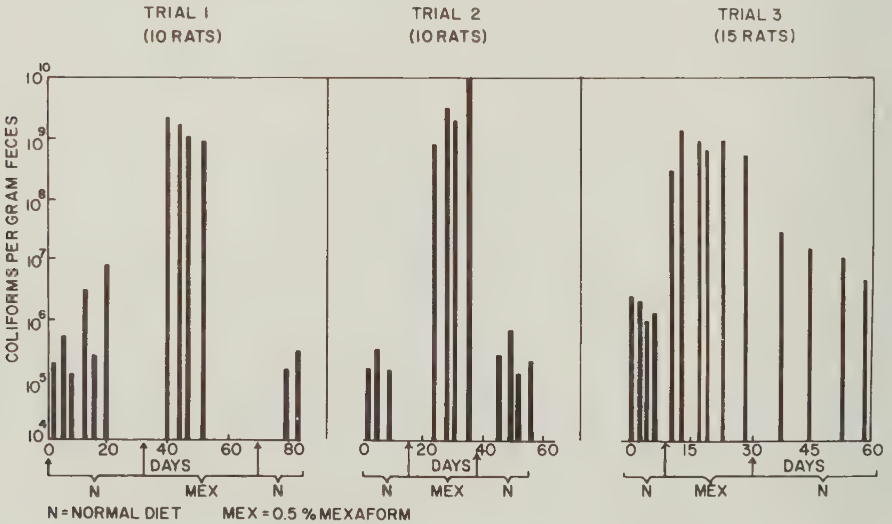


Fig. 2. Effect of Mexaform upon numbers of coliforms in rat feces.

agar, pH 7.2 (coliforms); and BBL mycophil agar, pH 7.0, supplemented with penicillin, 50 units/ml, and streptomycin, 100 μg/ml (yeasts). Ground Rockland diet was furnished ad libitum to the rats, this food being fortified, as required, with Mexaform (0.5%) or aureomycin (250 ppm).

Effect of Mexaform upon the total aerobic bacterial and coliform fecal flora of normal rats. The results of three separate trials expressed as average values are presented graphically in Fig. 1 (total aerobic bacteria) and Fig. 2 (coliforms). In each of the first two trials ten rats were used. While still on normal (unmedicated) food three fecal specimens, collected approximately four days apart, were plated for these organisms. Thereafter, during a period of five weeks when 0.5% Mexaform food was furnished to the animals, five samples were plated, the first being collected seven days after the commencement of medication. Normal food was then restored and, again, five samples were collected, approximately one week apart.

In the third trial, in which fifteen rats were employed, fecal specimens were collected on each of five successive days before the animals were given the Mexaform-containing food. During this period of medication (22 days) a total of 12 samples were plated. Upon reinstitution of normal food (36 days), six specimens were collected and plated for count.

Although variations in total bacterial and coliform counts occurred in individual rats throughout these experiments, the administration to normal rats of 0.5% Mexaform markedly increased their normal bacterial flora. During the five-week period of Mexaform feeding the counts of both total aerobic bacteria and coliforms increased by several thousandfold in the first two trials. Although no significant change occurred in the total aerobic count during Mexaform treatment in the third trial, the coliform level was significantly higher. In each of these trials reinstitution of normal food brought about a fall in the coliform counts to levels observed prior to medication with this drug, whereas the total aerobic bacterial count slowly decreased to the previously observed levels but not during the observation period.

Effect of Mexaform upon the coliform and yeast fecal flora of rats pretreated with chlortetracycline.* During a seven-day period and while the rats were still on normal food, fecal specimens were collected from 26 rats on each of five successive days and plated for yeasts and coliforms. The food was then replaced with chlortetracycline diet (250 ppm) and furnished to the animals for 38 days, during which time a total of 11 samples were plated. At the end of this period the animals were separated into three groups by random selection and treated with different diets for the ensuing 28 days, during which time five samplings were made. The groups are: a) chlortetracycline, 250 ppm plus Mexaform, 0.5% (9 rats); b) Mexaform, 0.5% (9 rats); and c) normal food (8 rats).

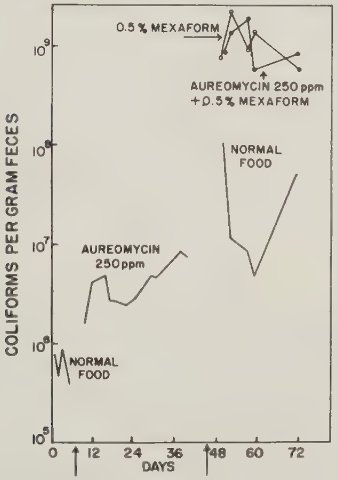


Fig. 3. Effect of Mexaform on coliforms in chlortetracycline-treated rats (average values).

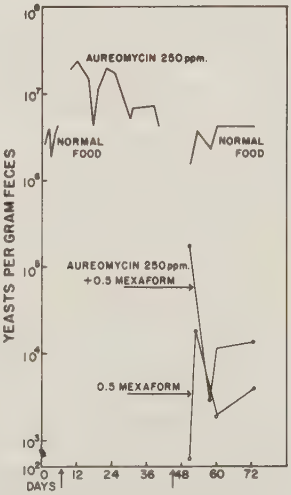


Fig. 4. Effect of Mexaform upon numbers of yeast in chlortetracycline-treated rats.

The results are presented graphically in Fig. 3 (coliforms) and Fig. 4 (yeasts), in which the average counts for each plating period are plotted. There occurred a four- and sevenfold increase in the numbers of yeasts and coliform organisms, respectively, as a consequence of chlortetracycline feeding.

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*The chlortetracycline was furnished by Lederle Laboratories, a division of American Cyanamid Company, Pearl River, New York.

Table 2. Effect of Mexaform upon Coliforms in Chlortetracycline-Treated Rats

Sequence of feedings	No. of Rats	Period of treatment, days	No. of samples plated	Average number per gram of feces	
				Coliforms $(\cdot 10^6)$	Yeasts $(\cdot 10^6)$
1. Normal food	26	7	5	0.6 (0.4-0.9)*	3.2 (1.5-4.2)
2. Chlortetracycline, 250 ppm	26	38	11	4.3 (1.7-8.6)	12.2 (4.2-23.6)
3. a) Chlortetracycline, 250 ppm plus Mexaform, 0.5%	9	28	5	1122 (590-1883)	0.05 (0.003-0.18)
b) Mexaform, 0.5%	9	28	5	1234 (620-2212)	0.006 (<0.001-0.019)
c) Normal food	8	28	5	37 (5.1-52)	3.2 (1.6-4.2)

*These values refer to the minimum and maximum plate counts.

An over-all summary of the data is shown in Table 2. Chlortetracycline-treated rats (group a) were receiving a diet containing 250 ppm chlortetracycline and 0.5% Mexaform demonstrated a remarkable increase in the coliform count of approximately 2000 times that existing in the rats at the beginning of this experiment. At the same time the yeast count fell to a level considerably below (1/60) that observed while the animals were on normal diet only. It is significant that these changes in fecal populations occurred in spite of the presence of chlortetracycline in the food. Similar results were observed in group b which received a diet containing only 0.5% Mexaform. However, these changes were more marked and occurred sooner than those observed in group a. The third group of rats (group c), which was given normal food following a course of chlortetracycline feeding, underwent a return to the original yeast count while the coliform count rose to levels slightly above those noted during the period of chlortetracycline feeding.

DISCUSSION

It is clearly evident from these experiments that Mexaform brings about a marked stimulation in the growth of coliform bacteria and a less marked although definite increase in total aerobic bacteria within the intestinal tract when incorporated into the food of rats. Although the data have not been presented here, there also occurs a simultaneous and significant decrease in the yeast population. This latter effect is in accord with the opinions expressed by other investigators [16, 19, 20] that the normal bacterial flora of the intestinal tract plays an important role in suppressing the growth of various pathogenic microorganisms, including C. albicans. If, indeed, the normal bacterial flora is capable of preventing the multiplication of these pathogens within the intestinal tract, it may be that any substance which brings about a marked increase in the numbers of a significant segment of the normal intestinal bacteria enhances the suppressive action of these organisms upon secondary invaders. This effect may be responsible for the favorable clinical reports regarding the chemotherapeutic activity of

Mexaform in various types of infectious enteritis. It is a reasonable working thesis that the ecologic changes brought about by Mexaform within the intestinal tract may create an environment in which yeasts and other microorganisms, whose establishment is associated with enteritis, are suppressed.

SUMMARY

The feeding of rats with 0.5% Mexaform in ground food resulted in a several thousandfold increase in the coliform count and a significant, but less marked, increase in total aerobic bacteria of the feces. Rats treated with chlortetracycline developed a high fecal yeast count. The addition of Mexaform to the chlortetracycline diet resulted in a considerable increase in the coliform population which was approximately 2000 times that existing in the rats at the outset of the experiment. Concurrently, the yeast count fell to exceedingly low levels. When the chlortetracycline diet was supplanted by a Mexaform diet, the results were similar except that the changes were more marked and occurred earlier. The findings support the view that the normal intestinal flora exerts an ecological barrier to the establishment and abnormal multiplication of certain pathogens and nonpathogens which may gain access to the intestinal tract. The clinical implications regarding the effects of Mexaform upon the suppression of secondary intestinal invaders have been briefly discussed.

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DISCUSSION

GEORGE GALE (American Cyanamid): Dr. Eisman, have you ever done any experiments of this sort with chicks or with any other animals besides rats?

DR. EISMAN: We are presently working with chickens, and it is a bit too soon to speak.

RELATIVE MERITS OF SERIAL DILUTION AND CUP-PLATE ASSAYS FOR DETERMINATION OF BLOOD LEVELS OF PENICILLIN, AND THE USE OF DIFFERENT DILUENTS IN BOTH METHODS*

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INTRODUCTION

With the advent of the new, partially synthesized penicillins it seems appropriate to re-evaluate methods of measuring penicillins in body fluids. There has been a tendency to interpret serum levels of unbound penicillin as a measurement of clinical potency, and claims of clinical superiority of at least two preparations of penicillin have already been made as a result of comparisons of their blood levels. It is essential to scrutinize carefully the techniques from which these questionable and conflicting conclusions have been drawn. In general two techniques for determining concentrations of penicillin in body fluids have been employed, the cup-plate assay and the serial dilution test. Many modifications in performance of each of these have been made in the years following their initial description by the Oxford group [1] and by Rammelkamp [2]. As long as benzylpenicillin was the sole penicillin each method of assay was considered acceptable despite variations in results which could be demonstrated when a single serum was tested by the two procedures [3]. Following the production of phenoxymethypenicillin (V), discrepant results, which were considered to be of clinical significance, were reported from different laboratories when blood levels of penicillins G and V were compared [4-6]. Subsequently, with the introduction of phenoxyethylpenicillin (152), there have been multiple studies which have claimed superiority of one over the other by comparisons of their blood levels as determined by a diversity of techniques [7-9]. This report summarizes a study which was designed primarily to partially explain these striking disparities in results of assays of blood containing various penicillins as performed by the two accepted methods for their measurement.

METHODS

Four penicillins were studied in human subjects: benzylpenicillin (G), phenoxymethylpenicillin (V), phenoxyethylpenicillin or phenethicillin (152), and 2,6-dimethoxyphenylpenicillin (1497). Concentrations of each penicillin were assayed at 1, 2, and 4 hr after the oral (G, V, 152) or intramuscular (1497) administration of a single dose. Each drug was given to essentially healthy ambulatory adults, whose ages ranged from 35 to 65 years, approximately 2 hr after the morning meal. Measurements of concentrations of each penicillin were determined by cylinder cup-plate method using Sarcina lutea (ATCC 9341) as the test organism and by the serial dilution technique using Staphylococcus 209 P as the test organ-

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*Work supported by grants from Bristol Laboratories, Inc., Syracuse, New York, and Parke Davis & Company, Detroit, Michigan.

ism. The techniques of the assays used have been described elsewhere [3, 10]. Although there were exceptions, each subject received in succession all four products one week apart. In each series predose blood was found to contain no antibiotic activity.

Normal serum, used as a diluent, was obtained from a serological laboratory. Daily batches were pooled from 10-25 individual specimens. The pooled material was tested for antibacterial content before it was used as a diluent and in no instance was it employed unless proved free of such activity. With refrigeration normal serum became more alkaline after 24 hr; it was adjusted to pH 7.4 immediately before use. The molecular equivalent dosage of the four drugs was approximately similar.

RESULTS

There is poor agreement in results with each penicillin when comparison of concentrations is made between the two assay methods in which the commonly employed 5% gelatin solution (cup-plate) and brain-heart infusion broth (serial dilution) are employed. Levels of each of the four products studied at 1 and 2 hr are shown in Table 1 (similar discrepancies are obtained at 4 hr, but not shown).

Table 1. Levels of Four Penicillins After 1 and 2 hr

Penicillin	Dose	Hour	Assay result,* μg/ml		
			Cup-plate		Serial dilution
			Normal serum	5% gelatin	Brain-heart infusion broth
G	300 mg	1	0.59	0.55	<0.33
		2	0.57	0.56	<0.3
V	250 mg	1	1.71	1.46	1.2
		2	1.73	1.41	1.5
152	268 mg	1	2.82	2.08	2.76
		2	3.48	2.54	2.98
1497	2 g	1	36.2	31.7	24
		2	28	20.2	19.2

*Average for 10 subjects with each drug.

Average levels observed with the serial dilution test are usually lower than those measured with either diluent in the cup-plate assay. For example, in the instance of penicillin 1497 at 1 hr the concentration is from 20 to 33% lower (24 vs 31.7 or 36 μg/ml). Levels of penicillin V, in general, are more comparable when measured by the two procedures than is evident with the other three penicillins (at 2 hr; 1.5 μg/ml with the dilution test, 1.41 and 1.73 μg/ml with the cup-plate). In the cup-plate method, levels using normal serum as the diluent are almost always higher than those with 5% gelatin or gelatin-phosphate combination as the diluent.

Following a single dose of a penicillin to a group of subjects, individual levels are quite inconstant, irrespective of the technique used to assay them. Examples of levels following the administration of penicillin 152 are shown in Table 2. Averages made from such diverse results must be interpreted with caution. Similar variations are observed at every hour with each penicillin and in both assays.

Table 2. Levels of Penicillin 152 (250 mg) 1 and 4 hr Following Administration

Subject	Assay results, $\mu g/ml$		
	Cup-plate		Serial dilution, brain-heart infusion broth
	Normal serum	5% gelatin	
	1 hr after administration		
1	5.4	4.4	8
2	5.6	4.7	8
3	1.75	1.3	2
4	2.8	2.4	4
5	2.25	1.9	2
Average	3.36	2.94	4.8
Average of another group of 5 subjects	3.86	2.46	2.35
	4 hr after administration		
1	0.18	0.09	<0.125
2	0.45	0.29	<0.125
3	0.17	0.08	0.125
4	5.3	4.4	8
5	1.05	0.8	1
Average	1.43	1.13	<1.95
Average of another group of 5 subjects	0.82	0.45	0.36

Variations in individual levels are marked regardless of drug or technique used to measure them.

In the serial dilution tests, missing the end point because of an improper dilution or because of interpretation makes average results more inaccurate.

Modifications of these two techniques were made to achieve a more standardized procedure for each method, and more consistent results between the two methods. Changes were first made in the diluents. Among a series of diluents thus studied, results with the following are judged important: in the cup-plate assay (a) normal serum containing no antibacterial activity, (b) 1.75% bovine albumin solution, (c) 5% gelatin solution, and (d) gelatin-phosphate buffer solution in a 1:2 proportion; in the serial dilution method (a) normal serum, and (b) brain-heart infusion broth. As staphylococci do not grow well in albumin solutions, they could not be utilized in the latter test. Results with gel buffer have been omitted from most of the tables in this report for the sake of simplicity and because gel buffer did not solve the problem under study. All diluents were adjusted to pH 7.4 before use.

The use of normal serum as the diluent is followed by more comparable results between the two assays in the instances of the two "synthetic" penicillins (152 and 1497). Table 3 shows both the individual levels at 1 hr in five subjects who received penicillin 1497 and the average levels in five other subjects (only three diluents are listed). It is apparent from the averages shown with 1497 that a comparison of the two techniques can be made when normal serum is used in both assay methods. Results with the use of normal serum in the two assays measuring 152 are also of the same order.

Table 3. Levels of Penicillin 1497 1 hr Following Administration

Subject	Assay results, μg/ml			
	Cup-plate		Serial dilution	
	Normal serum	5% gelatin	Normal serum	Brain-heart infusion broth
1	56.0	46.0	32.0	32.0
2	38.0	36.0	32.0	32.0
3	15.0	16.5	16.0	8.0
4	29.0	26.0	32.0	16.0
5	43.0	34.0	64.0	32.0
Average	36.2	31.7	35.6	24.0
Average of another group of 5 subjects	28.0	20.2	26.8	19.2

On the other hand, employing normal serum as the diluent, levels of penicillins G and V, as measured by the serial dilution technique are quite variable and results with it are not sufficiently akin to the cup-plate method utilizing any of the three diluents listed that any comparisons are warranted (Table 4). Despite studies with a number of diluents in assays of penicillins G and V, results of the serial dilution test are nearly always appreciably lower than those found with the cup-plate method.

Table 4. Levels of Penicillin V 1 hr Following Administration

Subject	Assay results, μg/ml				
	Cup-plate			Serial dilution	
	Normal serum	1.75% bovine albumin	5% gelatin	Normal serum	Brain-heart infusion broth
1	4.50	4.3	3.90	4.00	4.00
2	0.72	0.69	0.55	0.50	0.50
3	0.80	0.76	0.62	1.00	1.00
4	2.60	2.7	2.40	>2.00	>1.00
5	0.70	0.62	0.47	0.50	0.50
Average	1.86	1.81	1.59	>1.60	>1.40
Average of 5 other subjects	1.56	1.56	1.33	0.85	0.83

Only with synthetic penicillins and the use of normal human serum as the diluent has it been possible to make results by the two techniques at all similar.

Bovine albumin in a 1.75% solution is an adequate substitute for normal serum in the cup-plate procedure. It can be used with any of the four penicillins studied (Tables 4 and 5). In general, levels as measured with albumin are higher than those noted with 5% gelatin or gel-buffer solutions and are of the same order as are observed with serum as the diluent. As staphylococci do not grow well in albumin solutions it is not a substitute for serum in the serial dilution test; in fact, none of the many other diluents studied with this method is appreciably different from those obtained in brain-heart infusion broth.

The four penicillin preparations presumably bind with protein to different degrees. The extent to which penicillin 152 can bind to the protein in the diluent used in the cup-plate assay is shown in Fig. 1. Approximately similar standard

Table 5. Levels of Four Penicillins After 1 hr Using Cup-plate Assay and Different Diluents

Penicillin	Assay results,* μg/ml		
	Normal serum	5% gelatin	1.75% bovine albumin
G	0.59	0.55	0.60
V	1.71	1.46	1.69
152	2.82	2.08	2.84
1497	22.8	20.3	23.8

*Average for 10 subjects with each drug.

curves are also observed with penicillin V. Variations in zone size with each of four diluents at any given point in the curve are appreciable. For example, at the 0.2 μg/ml concentration, zone sizes range from 16.0 mm with normal serum to 21.8 mm with gel-buffer. In interpreting results of zone size in the unknown, disparities in results obviously must occur. Standard curves with normal serum and 1.75% bovine albumin are quite close, suggesting essentially similar binding of the penicillin to each.

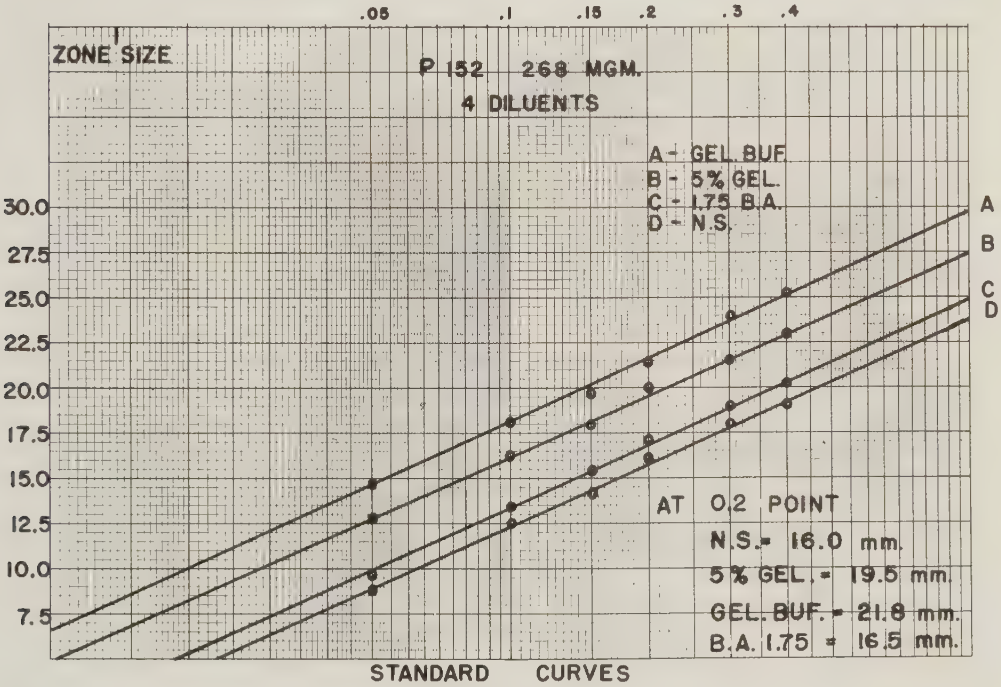

Fig. 1.

Binding of penicillins G and 1497 to protein must be of a different and probably lower order than that of 152 and V, because the standard curves of each are less widely separated for most of the diluents studied (Fig. 2). Table 6, for example, shows results in determination of penicillin G content of one subject's serum 1, 2, and 4 hr after a dose, as calculated from the standard curves depicted in Fig. 2. Each specimen of serum from this subject was divided into six aliquots. With the cup-plate method a narrow difference in concentrations occurs with

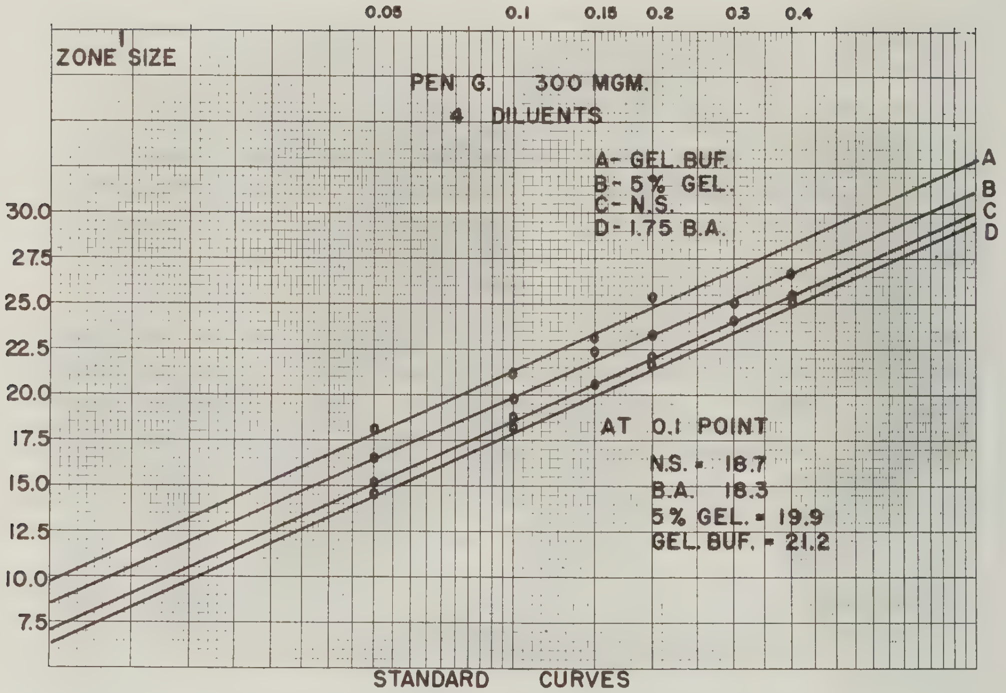

Fig. 2.

normal serum, 1.75% bovine albumin, and 5% gel. Zone sizes on the standard curves with these three diluents range only from 18.3 to 19.9 mm. Levels with gel-buffer as the diluent, on the other hand, are quite different as would be predicted from the standard curve.

Table 6. Various Levels of Penicillin G in Subject No. 1

Hour	Assay results, μg/ml					
	Cup-plate				Serial dilution	
	Normal serum	1.75% bovine albumin	5% gelatin	Gel buffer	Normal serum	Brain-heart infusion broth
1	0.46	0.46	0.40	0.37	1.0	0.25
2	0.26	0.23	0.20	0.16	0.25	0.25
4	0.10	0.12	0.08	0.07	0.125	0.06

DISCUSSION

Two clear impressions can be made from these observations: (a) Levels of any penicillin in blood as measured by two assays are sufficiently dissimilar that any comparison of results has little meaning or purpose. (b) A comparison of blood levels of two penicillins done by either or both assays similarly has neither significance nor value.

No method or modification has so altered or improved either of the two techniques for measuring penicillin such that results from one are exchangeable with results from the other. When measuring concentrations of a single penicillin by both methods it appears that two different substances are being assayed. Only using synthetic penicillins, with normal serum as the diluent, are the results of both procedures in some agreement, but this observation does not obtain with other preparations of penicillin.

We do not necessarily imply that one or the other of the two techniques for measuring penicillin content in body fluids should be discarded. Indeed with either method reproducible results can be obtained in one laboratory if the test is executed properly. On the other hand, it is evident that unless precisely the same techniques and identical diluents are utilized, results from one test, when performed in different laboratories, are no more analogous than the demonstrated disparate results obtained with comparison of the two assays. Protein binding of penicillin by the diluent conceivably plays a role in this discrepancy. Indeed protein binding is crucial in the final interpretation of blood-level results between drugs and between methods of assay, particularly when diluents containing different kinds of protein and in different concentrations are utilized. In our opinion this property greatly determines the inevitable result of any assay of penicillin. Standardization of this variable is essential. This can be achieved under some circumstances, and with some penicillins, by using a diluent containing protein like that occurring in vivo. Thus, we do consider the cup-plate method, with normal serum or 1.75% bovine albumin solution as the diluent, the accurate and appropriate test for practical clinical purposes. The serial dilution test nearly always gives results lower than those obtained with the cup-plate method and since protein binding is not taken into proper account we believe that these results are less realistic.

Another reason for the failure of the two assays to give similar results may be that the techniques specify different conditions for exposure of drug and test organism. Sarcina are incubated at 28 C (cup-plate) and staphylococci at 37 C (serial dilution). If the temperature of incubation does not permit maximal growth of the organism, an event which enhances penicillin antimicrobial activity, the killing effect of penicillin may be reduced. Diffusion of penicillin into the medium may also influence the results in the two assays [11, 12]. The serial dilution technique, using a rapidly diffusing liquid medium and incubating at a more nearly optimal temperature, theoretically should permit a more intimate exposure between the organism and the drug, therefore promoting a higher antibacterial effect of the penicillin. This is not so, and the paradox deserves further study.

If a single penicillin cannot be measured by two assays, it is highly unlikely that a comparison of levels after the administration of two different kinds of penicillin, with either or both techniques, could be meaningful. This is judged to be all the more important when variations in protein binding among the various preparations of penicillin are considered. The use of any test for the comparison of blood levels between two penicillins must, for clinical purposes, take into account protein binding in a manner that simulates or reflects in vivo conditions. This is not done as a general practice. Obviously a drug's efficacy in the treatment of an infection can only be judged by clinical trials with that infection; blood-level studies merely lead to an initial prescription of the penicillin. The comparative magnitude of blood levels does not intimate that one penicillin has more merit than another.

Finally, differences in absorption of a drug from the depot into which it is deposited vary appreciably. This is true particularly in the intestinal tract. Because no two penicillins can be expected to traverse the intestinal wall in the same fashion, it is not likely that resultant blood levels reflect accurately the clinical potency of that agent in any regimen of therapy. Moreover, differences in the chemical side-chains of the various penicillins, their respective shapes, weight, and size may further alter the physical factors responsible for both absorption and their measurement in vitro.

A suggestion has been made recently that "if various products are to be called penicillins, their activity should be indicated in terms of a standard unit such as that of penicillin G, as determined by acceptable standard biologic assays, in addition to indicating the content by weight of the molecular equivalent of the penicillin acid [9]." An analogous recommendation was made five years ago [6] but, fortunately, the suggestions to date have not been acted upon favorably. Actually, the idea of a single standard for all penicillins seems unnecessarily complicated and, in fact, might very well lead to incorrect assumptions in expressing clinical activity of any one of them. Assay methods determine only the amount of unbound penicillin in serum. As different preparations of penicillin presumably are bound to protein in different degrees, it does not seem proper to express antibacterial activity of serum when the penicillin used as the standard in the test binds in a different manner than the drug under consideration. The presumed efficacy of the measured penicillin content in serum can thus be measured accurately only if the penicillin given to the patient is also the standard used in the assay of his serum. This is particularly essential if normal serum is used as the diluent, a procedure which we highly recommend.

SUMMARY

There are marked differences in concentrations of four penicillins (G, V, 152, and 1497) when measured by the two generally accepted techniques for assaying blood levels. Comparison of levels of one penicillin measured by one of the tests in two laboratories is not valid unless identical procedures, including the use of the same diluent, are used. With either technique, individual levels are markedly variable, but, in general, levels measured by the cup-plate method are higher than those found by the serial dilution technique. Use of normal serum as the diluent in both assays yields more comparable results between the two assays in the instances of the two synthetic penicillins. When levels of penicillins G and V are measured, the serial dilution test, using normal serum as the diluent, gives results which are not very different than those observed with brain-heart infusion broth. However, levels with both diluents are appreciably less than those obtained in the cup-plate assay with normal serum. A 1.75% albumin solution is an adequate substitute for normal serum in the cup-plate method. Because different preparations of penicillin are bound to serum proteins to varying extents, a technique to measure unbound penicillin in serum must include the use of a diluent with a protein make-up closely simulating that of normal serum. A 1.75% bovine albumin in the cup-plate method achieves this purpose to a significant degree. Comparison of blood levels of any two penicillin preparations should not lead to conclusions about their relative clinical merits and, in fact, such comparison serves no useful purpose, and indeed may be misleading.

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PRODUCTION AND THERAPY OF SUBACUTE AND CHRONIC LISTERIOSIS IN EXPERIMENTALLY INFECTED MICE

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INTRODUCTION

Listeriosis has been found as an infectious disease of man and at least 36 species of animals in more than 30 countries. While ten years ago the world literature on this subject comprised less than 100 observations on proved occurrences of human listeriosis, the number of human cases alone has now reached almost 1000. In Germany, for instance, listeriosis in man was not described until 1951, but by 1956 some 300 cases had been detected by various investigators. During the first nine months of 1960, over 200 strains of Listeria monocytogenes were submitted for typing to the Listeria center operating at the Hygiene-Institute of the Bonn University and about 150 of these strains originated from fresh human cases observed in our country. This situation is paralleled by almost identical observations in other parts of Western, Central, and Northern Europe as well as on the North American continent.

This sudden increase during the past decade, however, does not mean that there has been, or still is, an actual increase of the actual listeriosis morbidity rate, which was probably no lower in earlier days. Early pathologico-anatomical literature contains numerous reports on cases which suggest Listeria infections described under other names such as "pseudotuberculosis," argentophile septicaemia, etc. [1-3]. The seeming increase in the number of reports of human infections due to L. monocytogenes is, in our opinion, due mainly to a better recognition of the causative agent and may just be a consequence of the awareness of the disease and of the better methods of detection. Yet there can be hardly any doubt that the impressive and rapidly growing figures represent only a tiny fraction of the Listeria infections which actually occur in all parts of the world.

A common feature of most of the pertinent reports on the finding of the disease among man and animals is that the observations were made mostly on acute cases which usually took a dramatic clinical course, often ending fatally. In recent years evidence has been accumulating which indicates that, in addition to acute to fulminant infections, subacute and chronic forms of the disease occur. This was first concluded from the results of serological investigations in groups of children suffering from cerebral disorders of unknown etiology [4-6] and later in women who had experienced repeated abortions [3, 7]. It has been known for a long time that Listeria may provoke dangerous infections of the central nervous system and that it is an important cause of abortion, stillbirth, and death in early childhood [1, 8, 9], but the hitherto published reports deal almost exclusively with acute cases only. Findings of French, American, and German workers [10-12] in natural cases and in experimental infections likewise suggest that subacute and chronic listeriosis may also occur in animals and that such infections may result in genital listeriosis with abortion through several succeeding generations. As was recently pointed out [13], such a series of abortions may lead to eventual sterility. Although no direct proof exists so far that listeriosis is a cause of so-called habitual abortions in pregnant women, it becomes more and more likely that such a condition, similar to that in animals, might prevail.

The beneficial effects of chemotherapeutic and antibiotic treatment observed in women suffering from repeated abortions [7] made it desirable to investigate the question of whether subacute or chronic Listeria infections could be produced experimentally and how such infections would respond to therapy. As the same clinical states which are found among animals are characteristic of human listeriosis as well, the results of such studies may serve as a useful guide in the treatment of subacute or chronic human infections. Furthermore, studies were contemplated in order to discover whether a clinical cure of infected animals is accompanied by the disappearance of the infectious agent from the body since it had been reported that the internal organs of mice, which under appropriate treatment had survived a lethal infectious dose, still harboured live Listeria several weeks after therapy [16]. Last, but not least, it was hoped that the investigations would determine which cultural methods were most suitable to prove the complete absence of living organisms in the tissues for recent experiences in the study of human Listeria infections have shown that the usual culture methods are insufficient and that the use of the technique suggested by Gray et al. [17] increases the rate of isolation of Listeria from specimens submitted for examinations [18, 19].

MATERIALS AND METHODS

Test strain. The present study was performed with a strain of L. monocytogenes 613, which belonged to serotype 1 and was isolated from the spinal fluid of a human case of meningitis. The strain was chosen at random from our Listeria culture collection and was maintained in nutrient agar stabs. During the investigation it was passed serially through mice. After intraperitoneal infection $46 \cdot 10^6$ organisms killed white mice, strain Agnes Blum, weighing 25 to 30 g, within 26 to 32 hr.

Choice of infectious dose. In a series of preliminary experiments, the details of which have been described [20, 21], conditions were sought under which early death due to the infection could be avoided. When the infectious dose was lowered to $2.3 \cdot 10^6$ organisms, only a very small percentage of infected mice died of acute listeriosis during the first 5 days and only a few animals succumbed to the infection in 6-23 days. The overwhelming majority of the animals survived the 24th day. Although the animals appeared normal, the livers of most of them still contained Listeria as was proved in 17 out of 19 mice which were sacrificed from 25 to 45 days after infection. Histological examinations showed tissue changes typical for listeriosis, many of them in the process of healing (Fig. 1). A detailed report on this will be presented later. Further investigations showed that most of the animals surviving 25 days remained alive for 87 days. Bacteriological studies of the livers of these animals revealed that they continued to harbor L. monocytogenes without apparent symptoms of the disease. From this it was assumed that the infection had passed from the subacute state, characterized by the development of listeriomas in the liver without subsequent death, to a chronic state lasting for almost three months. Therefore, an infectious dose of $2.3 \cdot 10^6$ organisms of L. monocytogenes 613 was used throughout the experiments for the production of subacute and chronic listeriosis in white mice.

Choice of therapeutic agents. Based on the results of therapy in acute Listeria infections of animals and man, which are summarized in the monograph of the senior author [3], the following antibiotics were used in this study: procaine-

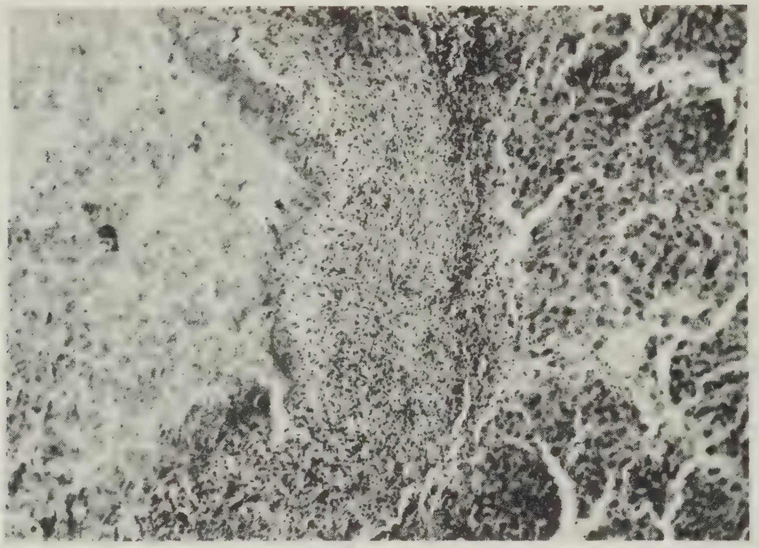

Fig. 1. Listerioma in liver section of mouse taken on the fifth postinfectious day.

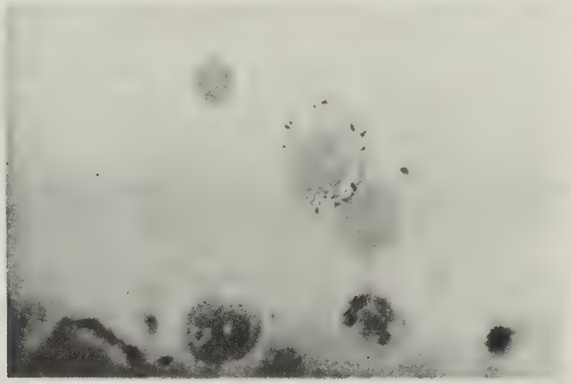

Fig. 2. Phagocytosis of L. monocytogenes in mouse peritoneal fluid (Giemsa stain).

penicillin (PP), Omnacillin (trade mark), procaine-penicillin in combination with dihydrostreptomycin (DSM), tetracycline (Achromycin was used in most experiments), and erythromycin. Therefore, besides penicillin and a penicillin-streptomycin combination, a middle-spectrum and a broad-spectrum antibiotic were included. All drugs were obtained from commercial sources and were prepared freshly for use according to the directions given by the producers. Furthermore, the effects of Omnadin (trade mark for a drug consisting of a mixture of protein and lipoid substances, bile salts, and animal lipoids, used for mobilization of the defense mechanisms) [14] were investigated for the following reasons. During experimental studies [15] distinct phagocytosis of Listeria was observed in the peritoneal fluid of mice after i.p. infection. This phagocytosis was pronounced on the third to fifth postinfectious days, i.e., when the signs of an acute infection subsided and there was a transition to clinical improvement. As numerous reports deal with the beneficial effects of "Omnadin" in subacute and chronic bacterial infections [14] it was considered worthwhile to try this drug alone and in combination with antibiotics in subacute and chronic listeriosis of mice since

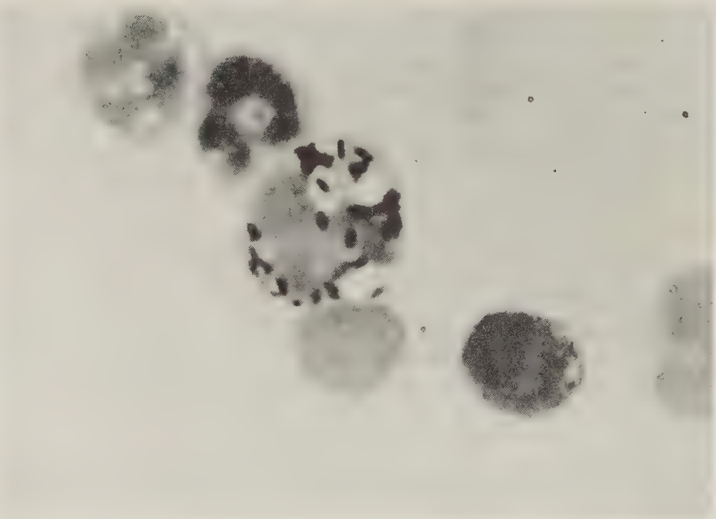

Fig. 3. Phagocytosis of L. monocytogenes in mouse peritoneal fluid (Giemsa stain). Higher magnification.

recent reports on the influence of this drug in the therapy of Erysipelothrix infections of mice have given conflicting results [22, 23].

The therapeutic scheme was quite different from that used in previous investigations for treatment of acute experimental listeriosis of mice in which the drugs were usually administered immediately after infection. In this study, the institution of treatment was delayed intentionally. In the first series of experiments on subacute listeriosis the drugs were injected subcutaneously 2, 3, 7, 8, 11, 13, 21, and 22 days after infection. In the second series of experiments, dealing with the treatment of chronic listeriosis, no animal was treated for 29 days following infection. Treatment was started after 30 days and was continued daily for 5 days with tetracycline and for 12 days with Omnadin. A second course of tetracycline treatment was then given to a number of animals in the period from 57 to 62 days following infection. The dosage is summarized in a following section.

Bacteriological methods. All animals which died spontaneously or which were sacrificed at various intervals during the observation period were subjected to careful bacteriological studies. If death occurred during the first 9 days following infection, the liver, spleen, heart, blood, and peritoneal washings were cultured. Animals dying later usually harbored the organisms in the liver only and therefore, only the liver was removed aseptically, ground up with sterile sand to a homogenous paste, and cultured. Part of this material was plated directly on blood and tellurite agar, some was inoculated into dextrose-tryptose broth with subsequent plating on blood agar after 24 hr incubation at 37 C, and the remainder of the tissue homogenate was placed in the refrigerator at 4 C after mixing with dextrose-tryptose broth. Subcultures were made after 14 and 28 days standing in the refrigerator (Gray's enrichment technique). All suspicious colonies were isolated in pure culture and tested biochemically and serologically according to the procedures outlined for the detection of L. monocytogenes [3]. In a number of instances the organisms were tested for drug sensitivity in vitro by means of the agar-diffusion method with filter-paper disks impregnated with 20 μg of tetracycline, streptomycin, erythromycin, or 1 unit of penicillin.

Evaluation of results. Since death due to the experimental infection was very rare and clinical symptoms of the disease were only transient if present at all, the effect of the treatment was controlled bacteriologically. Therefore, the efficiency of the drugs was evaluated from the rate of bacteriological cures in groups of the same size of treated and untreated animals. The percentages of positive and negative findings were checked for statistical errors according to the formula of Just,

$$m = \pm \sqrt{\frac{(p_0\%)(p_1\%)}{n}}$$

where m is the mean deviation, $p_0\%$ is the percentage of bacteriologically positive animals, $p_1\%$ is the percentage of bacteriologically negative animals, and n is the number of animals in the respective group.

For the calculation of the differences of the mean deviation the formula of Koller was applied,

$$\sigma_{\text{diff.}} = \sqrt{\sigma M_1^2 + \sigma M_2^2}$$

where $\sigma_{\text{diff.}}$ is the statistical error of the differences, σM_1 is the mean deviation of the value obtained with antibiotic therapy alone, and σM_2 is the mean deviation of the value obtained with antibiotic plus Omnadin therapy.

RESULTS

Subacute Listeria infections of mice.* In this part of the study, groups of 25 mice each were treated in the following manner: Group 1, control group, no treatment; group 2, Omnadin 0.5 ml diluted 1:5 (total dose 0.8 ml); group 3, PP 500 units (total dose 4000 units); group 4, PP 500 units plus Omnadin, 0.5 ml diluted 1:5 (total dose as in groups 2 and 3); group 5, Omnacillin 500 units (total dose 4000 units); group 6, PP—DSM 1:1, 500 units (total dose 4000 units); group 7, PP—DSM, 1:1, 500 units plus Omnadin, 0.5 ml 1:5 (0.8 ml) (total dose as in groups 2 and 6); group 8, tetracycline 500 μg (total dose 4000 μg); group 9, tetracycline 500 μg plus Omnadin 0.5 ml diluted 1:5 (total dose as in groups 2 and 8); group 10, erythromycin 500 μg (total dose 4000 μg); group 11, erythromycin 500 μg—Omnadin 0.5 ml diluted 1:5 (total dose as in groups 2 and 10). Details on the treatment were given in the preceding section. The number of deaths in these groups is compiled in Table 1; L. monocytogenes was isolated in pure culture from the livers of all those animals.

It can be seen that deaths occurred in all groups with the highest percentages in groups 1-4.

All animals surviving 25 days following infection were sacrificed on the 26th day. The results of the bacteriological studies performed with the livers of the animals which died spontaneously or were killed are summarized in Table 2.

Listeria could not be detected in 20% of the animals in groups 1 and 2. This might indicate either spontaneous cures or too low an infectious dose. There is also the possibility that the organisms may have been present in other tissues or that they may have escaped detection due to inadequate culture methods.

The highest rate of bacteriological cures was obtained after administration of tetracycline. This was followed by erythromycin and a combination of penicillin and dihydrostreptomycin. On the other hand, the rate of bacteriological cures after administration of Omnadin equalled that of the untreated control group.

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*This part of the report has also been published in Ärztl. Forschg. 13: 581-586, 1959.

Table 1. Deaths Due to Experimental Listeria Infections in 11 Groups of 25 Mice With and Without Treatment

Group	Deaths	Postinfectious day of death
1	3	6, 8, 10
2	3	8, 9, 10
3	5	7, 9, 10, 11, 13
4	3	9, 10, 12
5	2	7, 8
6	1	5
7	2	6, 7
8	2	8, 9
9	1	9
10	1	4
11	1	5

Table 2. Rate of Bacteriological Cures in Subacute Listeria Infections of Mice

Group	Treatment	Isolation of Listeria		
		Positive, %	Negative, %	Mean deviation
1	None	80	20	±8
2	Omnadin	80	20	±8
3	Procaine penicillin	68	32	±9.3
4	Procaine penicillin and Omnadin	56	44	±9.9
5	Omnacillin	64	36	±9.6
6	PP-DSM combination	32	68	±9.3
7	PP-DSM combination and Omnadin	36	64	±9.6
8	Tetracycline	16	84	±7.3
9	Tetracycline and Omnadin	12	88	±6.5
10	Erythromycin	36	64	±9.6
11	Erythromycin and Omnadin	28	72	±9.2

Table 3. Comparison of Bacteriological Cures After Antibiotic Therapy With and Without Omnadin

Drug	Cures, %		Difference, %
	Antibiotic therapy	Antibiotic therapy plus Omnadin	
Procaine penicillin	32 ± 9.3	44 ± 9.9	12 ± 13.6
PP – DSM	68 ± 9.3	64 ± 9.6	4 ± 13.4
Tetracycline	84 ± 7.3	88 ± 6.5	4 ± 12.5
Erythromycin	64 ± 9.6	72 ± 9.2	8 ± 13.1

A comparison of the results obtained with antibiotic therapy alone and with antibiotic therapy supplemented by administration of Omnadin is presented in Table 3.

Although the percentage of bacteriologically negative findings was greater in three groups of animals treated with Omnadin in addition to the antibiotics, a statistical evaluation of the differences shows they are still in the range of experimental error and, therefore, statistically insignificant.

L. monocytogenes was identified in 140 cultures after direct plating as compared to 172 positive cultures when Gray's cold enrichment was used. The superiority of the latter procedure is obvious, for the yield of positive isolations was 22.8% higher with this procedure than with direct cultivation.

Chronic Listeria infections of mice. In a subsequent investigation the observation time was prolonged to 87 days. One hundred mice were used in this study. The infectious dose was the same as in the previous experiment but no treatment was given during the first 28 days following infection. Twenty-six animals died spontaneously from acute and subacute listeriosis, and four mice were sacrificed after 30 days. Cultural examinations yielded L. monocytogenes in 28 instances. Listeria could not be obtained in pure culture from the livers of two animals due to overgrowth of other organisms but Gram-stained smear preparations showed the presence of organisms indistinguishable from Listeria. As the two animals died in the first half of the observation period it is safe to assume that their death was due to the infection. The death rate is summarized in Fig. 4.

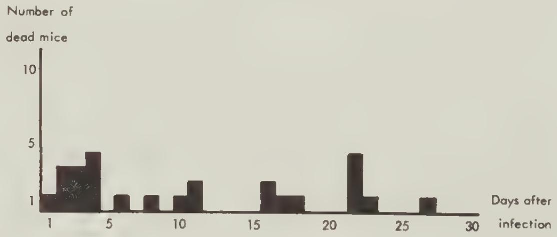

Fig. 4. Cases of spontaneous death in a group of 100 mice infected with $2.3 \cdot 10^6$ organisms.

The remaining 70 animals were divided into three groups. The first group, consisting of 20 mice, remained untreated; the second group (25 mice) received six 1.0 ml injections of the 1:5 dilution of Omnadin every second day; and the third group (25 mice) was treated daily with 500 μg of tetracycline subcutaneously for 5 days.

At 57 days after the infection, four animals were sacrificed in the first group and five animals in the second. From the livers of eight of these mice, L. monocytogenes could be isolated in pure culture. This indicated that despite the normal appearance and behavior of the animals and the lack of any symptoms which could be related to listeriosis the infection was still present in a chronic form. On the other hand, four of the five killed animals in the third group treated with tetracycline were Listeria-negative and the organism could be cultivated from one liver only. It may be added that treatment with tetracycline had been discontinued after 52 days so that the drug certainly did not interfere with the culture procedure. Although in this series the administration of tetracycline was extended to 5 days instead of 2 days, as in a previous experiment, the drug was not able to sterilize the livers of all treated animals when the treatment was delayed for 30 days.

Table 4. Treatment Schedule of 56 Mice

Group	Treatment schedule
1 16 mice, untreated	9 mice were not treated 7 mice received tetracycline for 5 days, 500 μg daily
2 20 mice, previously treated with Omnadin	10 mice received 6 doses of 1.0 ml of Omnadin in a dilution of 1 : 5, every second day 10 mice received tetracycline for 5 days, 500 μg daily
3 20 mice, previously treated with tetracycline	10 mice were not treated 10 mice received tetracycline for 5 days, 500 μg daily

Subsequently the remaining 56 animals of the 3 groups were divided into two subgroups which were treated as outlined in Table 4.

Only one of these 56 animals died spontaneously during the observation period which was extended to 87 days following infection. The dead animal belonged to the untreated group and death occurred after 63 days. L. monocytogenes was cultured from the liver. All other mice were sacrificed at 87 days. Cultural examinations of the livers yielded the results presented in Fig. 5.

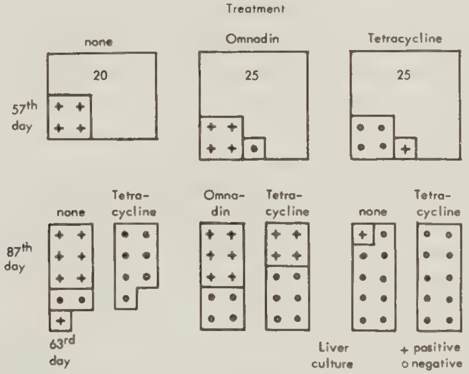

Fig. 5. Effect of specific and nonspecific treatment on the viability of Listeria in chronic listeriosis of mice.

It is obvious that the greatest number of positive findings occurred in the group which remained untreated during the whole observation period and that only two out of nine mice yielded negative findings. The percentage corresponds well with the earlier findings in subacute listeriosis reported above. Likewise six out of ten mice which received Omnadin were Listeria-positive on the 87th day, but four were negative. As the number of animals investigated was too small in this series, it is difficult to draw any conclusions as to the value of this kind of treatment although it seems that the application of Omnadin in chronic listeriosis of mice gave better results than in experiments with a shorter observation time.

The superiority of the administration of tetracycline is clearly indicated by the results obtained. All animals which received two courses of tetracycline were freed of the infection and bacteriologically cured. The same applies to a group of seven previously untreated mice which received one course of tetracycline from 57 to 61 days after infection. Of the animals which had received tetracycline from the 30 to 34 days after infection and had remained untreated thereafter, one still contained L. monocytogenes in the liver after 87 days. This is noteworthy since the late administration of tetracycline after a previous course of Omnadin treatment was unable to prevent growth of L. monocytogenes in four out of ten mice.

All negative findings reported in this series of experiments relate to direct cultures and to cultures after cold enrichment.

DISCUSSION

The foregoing experiments have shown that with an appropriate infectious dose of L. monocytogenes subacute and chronic infections of mice can be obtained. These observations confirm and supplement earlier investigations mentioned above. It is very likely that such subacute and chronic infections also occur under natural conditions and this might explain acute outbreaks or sporadic occurrences of listeriosis in animals under conditions of stress such as shipment over long distances, change-over in feeding, or sudden climatic changes. In such conditions latent or chronic Listeria infections might become acute. It seems probable that such latent infections are widely spread among animals and it may well be that a similar condition exists in man. Large-scale serological studies conducted in the author's laboratory are in good agreement with such a hypothesis.

The determination of the curative effects of antibiotics in experimental subacute and chronic listeriosis of mice presents certain difficulties. The frequency of death due to the infection was so low in the control groups that prevention of death could not be used as a criterion of successful treatment. The lack of evident clinical symptoms during the subacute or chronic stages of the infection likewise forbade a critical evaluation of the therapeutic effects. Therefore the efficiency of various drugs was tested in this study by the rate of bacteriological cures, i.e., treatment was considered effective only when cultures of the livers removed aseptically from the dead animals, did not contain viable Listeria. However, this preconditions reliable methods of cultivation of L. monocytogenes from tissue specimens.

It is generally accepted that the bacteriological methods of Listeria isolation are far from being satisfactory and this was borne out again in this study. The rate of positive findings was increased by more than 22% when Gray's cold-enrichment method was used. Therefore it is strongly recommended that this procedure be used routinely whenever attempts are made to culture L. monocytogenes. Of course cold enrichment is not the final answer to the unsolved problem of selective Listeria cultivation. It seems to be the method of choice with specimens which are not heavily contaminated with other organisms, as was usually the case in this study. The rate of positive findings from contaminated specimens is much lower since other bacteria such as Proteus and enterococci also multiply at low temperatures.

In our investigations, tetracyclines exerted a remarkable effect on the viability of L. monocytogenes in tissues and in this respect they are superior to all other drugs tested. Although the total dosage administered during the two series of experiments differed, the results of bacteriological cures were almost identical. Usually more than 80% of the animals treated with tetracyclines became Listeria-negative. The results obtained indicate that a more intensive treatment, such as was given in the second experiment, will not result in a much higher rate of cures than was obtained by intermittent therapy. However, a single course of tetracycline usually did not result in the killing of L. monocytogenes in all instances.

The reasons for this are not yet sufficiently understood. Perhaps in some cases the defense mechanisms of the body may be responsible for the failures observed. Development of encapsulated listeriomas may, for instance, prevent the drug from penetrating into the interior in sufficient amounts to exert an antilisteric effect.

The administration of Omnadin, a non-specific compound which increases the rate of phagocytosis and which may exert a beneficial effect in chronic bacterial infections, did not significantly alter the subacute listeric infection of mice. This corresponds to similar experiences in experimental Erysipelothrix infections [25]. No definite conclusions can be drawn with regard to the findings in chronic Listeria infections of mice since the number of animals appears to be too small.

The results of therapeutic studies in subacute and chronic Listeria infections of mice may indicate the therapeutic scheme to be followed in suspected cases of subacute or chronic human cases, particularly in chronic listeriosis of the female genital tract. As a matter of fact, tetracyclines have repeatedly been administered successfully in cases of habitual abortions which, according to serological tests, were of probable listeric origin [7]. These results were supported strongly by recent bacteriological findings in Israel [24, 25].

From previously gained clinical experience it has become evident that a single course of tetracycline does not always suffice to eradicate a proven or suspected listeric infection and it was found that two or more courses of tetracycline treatment may be necessary or that tetracycline treatment should be supplemented by the long-term administration of sulfonamides. The experimental results in chronic listeriosis of mice are in good agreement with this clinical experience for it was observed that all animals were bacteriologically cured after two courses of tetracycline.

Although many problems related to chronic Listeria infections still remain unsolved, it is hoped that this experimental contribution will serve as a stimulus to others approaching this interesting and promising subject.

SUMMARY

By administration of an infectious dose of $2.3 \cdot 10^6$ organisms of Listeria monocytogenes 613, subacute and chronic listeriosis was produced in white mice. In a first series of experiments the therapeutic value of specific antibiotic treatment was compared to nonspecific treatment of subacute Listeria infections of mice. The percentage of bacteriological cures was highest with tetracycline, followed by erythromycin and a penicillin-dehydrostreptomycin combination. In a second series of experiments with chronic Listeria infections of mice, tetracycline again effected a bacteriological cure in the overwhelming majority of the treated animals after observation periods of 57 and 87 days. The results are discussed in relation to the antibiotic therapy of suspected or proven cases of human listeriosis.

In bacteriological studies, Gray's cold-enrichment method was found to be superior to direct-culture procedures.

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THE DIFFERENTIATION OF DEGREES OF SENSITIVITY TO VARIOUS PENICILLINS BY USE OF SINGLE α-PHENOXYETHYLPENICILLIN DISCS

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INTRODUCTION

Surveys [1-4] have shown that paper discs impregnated with antibiotics or tablets containing these drugs are used most widely in clinical laboratories for detecting the degree of sensitivity of bacteria. Many observers [5-7] have also published data demonstrating that the disc method may yield reproducible results even to a quantitative degree of accuracy if the concentration of antibiotic in the disc is uniform and the techniques employed are carefully standardized. This applies to the majority of antibiotics now in common use, although it is doubtful whether this method can be expected to furnish satisfactory results with rapidly diffusible drugs such as cycloserine or with very slowly diffusible drugs like polymyxin. The regression lines in the former are too steep and in the latter too flat for the gradients to yield the necessary differentials. In the case of penicillin G, when testing staphylococci which owe their resistance to their production of the enzyme, penicillinase, the difficulty of ensuring accuracy is due largely to variations in penicillinase production. There are, however, properties inherent in this antibiotic which place these discs in a special category. Penicillin G is very hygroscopic and unstable and the stored vials must be constantly refrigerated and opened as infrequently as possible to avoid deterioration. Once opened the discs should only be used for a short time as the potency of the antibiotic in the disc is rapidly reduced. Also, the speed of the destruction of the rapidly diffusing penicillin G by penicillinase tends to cause an inconstancy in the results of the tests with penicillinase-producing staphylococci.

The first synthetic penicillin available, α-phenoxyethylpenicillin or phenethicillin (Syncillin),* is more stable, more slowly diffusible, and less rapidly destroyed by penicillinase than penicillin G. For these reasons discs made from this drug were tested against a panel of staphylococci with known varying resistance to penicillin G to determine whether the inconstant results obtained with penicillin-G discs could be obviated.

METHODS

Thirty-five strains of coagulase-positive staphylococci isolated from human infections were employed, the sensitivities varying from 0.19 to > 200. Manufactured phenethicillin discs, labeled 2, 5, and 10 μg, were obtained from the Bristol Laboratories who assayed them to contain 2.4, 5.5, and 10 μg per disc, respectively. Penicillin-G discs of one manufacturer with labeled concentrations of 2 and 10 units were also tested. These had complied with the Canadian Government regulations but the actual assayed content was not known.

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*Trade name of Bristol Laboratories for α-phenoxyethylpenicillin.

For the disc tests, tryptose agar plates of a uniform depth were inoculated with 0.02 ml of an 18-hr culture of staphylococci previously diluted with tryptose broth to the opacity of the MacFarland Nephelometer tube No. 2, equivalent to $6 \cdot 10^8$ organisms/ml. The inoculum was spread uniformly, the discs applied, and the plate incubated at 37 C for 18 hr. An almost confluent growth is obtained. In accordance with the recommendations of the Expert Committee on Antibiotics of the World Health Organization the size of the zone of inhibition was read in millimeters and recorded [4].

In the tube-dilution tests three penicillins, penicillin G, phenethicillin, and 2,6-dimethoxyphenylpenicillin (Staphcillin)*, were employed using twofold dilutions of the antibiotic in tryptose broth, the maximum concentration being 100-200 units/ml for penicillin G and 100-200 μg/ml for the other two. At the start of incubation there were $6 \cdot 10^5$ organisms/ml present. This inoculum was prepared from an 18-hr culture in tryptose broth. A second series of tests by the tube-dilution method was also performed on a selected number of these strains using a heavy inoculum. This consisted of 0.02 ml of undiluted culture in 2.5 ml of broth estimated to contain $12 \cdot 10^6$ organisms/ml or 20 times more than that described previously. The maximum concentration of antibiotic for this series was 500 units/ml for penicillin G and 500 μg/ml for the other two penicillins with two-fold dilutions. The tests were read in a constant light source and the lowest concentration showing no growth at the end of the incubation period was the minimum inhibitory concentration. All tests were performed by one individual.

RESULTS

The results of the tests with the standardized tube-dilution method with penicillin G, phenethicillin, and 2,6-dimethoxyphenylpenicillin, as well as the zone sizes of the three phenethicillin and two penicillin-G discs, are given in Table 1. Four strains were sensitive and these did not produce penicillinase by the Gots test. All the others produced penicillinase.

Table 1 illustrates several points of interest. Both the penicillin G and phenethicillin discs accurately outlined sensitive organisms. The 2-, 5-, and 10- μg discs of the latter all gave zones of inhibition of over 30 mm with the nonpenicillinase-producing organisms and smaller zones with any penicillinase-producing strain. The penicillin-G discs were not as consistent differentiating the low and high penicillinase producers, i.e., the resistant and moderately resistant organisms. Several moderately resistant strains gave no zones with the 10-unit disc. The three phenethicillin discs enabled a differentiation to be made between these three categories. Thus, with strains most resistant to penicillin G the 2-μg disc produced zones of 10-14 mm; the 5- μg disc gave zone sizes of 11-16 mm; and the 10- μg disc yielded zone sizes of 14-17 mm. The organisms moderately resistant to penicillin G show greater zones of inhibition with the same phenethicillin discs, 14-19 mm with the 2- μg disc; 15-20 mm with the 5- μg disc; and 17-22 mm with the 10- μg disc. The point at which a transition may be outlined occurs when organisms are sensitive to more than 3-5 units of penicillin G by the tube-dilution method. It should be noted that with the inoculum employed in these experiments highly resistant organisms show a small zone size with phenethicillin discs of all three concentrations. Manufacturers basing their interpretation of resistance on the absence of any zone should take account of this in issuing information pertaining to interpretation of the in vitro sensitivity tests. In order to show that the critical point outlined above by zone size may

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*Trade name of Bristol Laboratories for 2,6-dimethoxyphenylpenicillin.

Table 1. Comparative Tube-Dilution Sensitivity Tests by a Standardized Method Using Penicillin G, Phenethicillin, and 6-Dimethoxyphenylpenicillin

Strain No.	Sensitivity to Penicillin G*	Tube-dilution minimum inhibitory concentration			Disc zone size, mm				
					Phenethicillin			Penicillin G	
		A †	B	C	2 μg	5 μg	10 μg	2 units	10 units
1	S	0.19	0.19	1.56	30	33	34	15	17
2	S	0.19	0.19	0.78	31	33	35	18	22
3	S	0.19	0.39	1.56	32	34	36	24	31
4	S	0.19	0.19	1.56	30	33	35	25	31
5	MR	0.39	0.19	0.39	19	20	22	8	12
6	MR	0.39	0.39	0.39	17	18	20	0	13
7	MR	0.78 0.78 3.12	0.19	1.56	15	16	17	0	10
8	MR	1.0	0.19	1.56	19	0	21	0	0
9	MR	1.56	0.39	1.56	15	16	18	0	0
10	MR	1.56	0.19	1.56	15	16	19	0	10
11	MR	2.5	0.19	1.56	16	18	20	0	0
12	MR	2.5	0.39	1.56	15	16	18	0	8
13	MR	2.5	0.39	1.56	15	16	18	0	11
14	MR	2.5	0.39	1.56	15	17	20	0	0
15	MR	3.12	0.19	1.56	17	19	20	0	13
16	MR	3.12	0.19	1.56	17	20	21	12	16
17	MR	3.12	0.39	1.56	15	17	19	14	15
18	MR	3.12	0.19	1.56	14	15	18	9	10
19	R	5.0	0.39	0.78	14	15	16	0	0
20	R	25.0	1.56	3.12	11	11	14	0	0
21	R	25.0	0.78	1.56	14	14	17	0	0
22	R	25.0	0.39	1.56	14	16	17	0	11
23	R	50.0	0.78	1.56	11	14	15	0	8
24	R	50.0	3.12	1.56	12	14	15	0	0
25	R	50.0	1.56	3.12	12	14	15	0	0
26	R	50.0	1.56	1.56	13	14	16	0	0
27	R	>50.0	3.12	1.56	10	11	14	0	10
28	R	100.0	6.25	1.56	11	14	15	0	0
29	R	100.0	3.12	1.56	13	15	15	0	0
30	R	100.0	6.25	1.56	10	15	16	0	0
31	R	100.0	6.25	1.56	13	14	16	0	0
32	R	>100.0	3.12	1.56	12	14	15	0	0
33	R	>100.0	3.12	1.56	13	14	15	0	0
34	R	200.0	6.25	1.56	10	12	14	0	0
35	R	200.0	6.25	3.12	12	13	15	0	0

*S, sensitive; MR, moderately resistant; R, resistant.

†A=penicillin G, units/ml; B=phenethicillin, μg/ml; C=2,6-dimethoxyphenylpenicillin, μg/ml.

have some bearing on the degree of penicillinase production, 17 strains were selected from the panel of organisms listed in Table 1 covering the various degrees of resistance and the tube-dilution tests were repeated with a heavy inoculum [8, 9] against penicillin G, phenethicillin, and 2,6-dimethoxyphenylpenicillin.

Table 2 shows that the minimum inhibitory concentration of 2,6-dimethoxyphenylpenicillin is not changed for either sensitive or resistant organisms. In the case of organism 9 (No. 19 of Table 1), sensitive to 5 units of penicillin G and 0.39 μg of phenethicillin with the smaller inoculum and giving a smaller zone with phenethicillin discs than more sensitive organisms, resistance has increased to a point where the organism is sensitive to 31.25 μg of phenethicillin. This increase did not occur in less resistant organisms but was constant in all the staphylococci of greater resistance. In our opinion this may indicate that staphylococci showing resistance to more than 3 units of penicillin G are untreatable with phenethicillin.

Table 2. Comparison of Tube-Dilution Sensitivity Tests Using Standard and Large Inocula

Strain No.	Minimum inhibitory concentration					
	Penicillin G		Phenethicillin		2,6-Dimethoxyphenylpenicillin	
	Standard, units/ml	Large, units/ml	Standard, μg/ml	Large, μg/ml	Standard, μg/ml	Large, μg/ml
1	0.05	0.06	–	0.03	–	0.97
2	0.39	50	0.19	3.12	–	–
3	0.39	25	0.39	1.56	–	–
4	1.0	25	0.19	3.12	–	–
5	1.56	15.6	0.19	7.8	1.56	1.95
6	2.5	100	0.39	3.12	–	–
7	3.12	50	0.19	3.12	–	–
8	3.12	100	0.19	3.12	–	–
9	5.0	125	0.39	31.25	–	–
10	25	500	0.39	62.5	1.56	1.95
11	25	>500	0.78	500	1.56	0.97
12	>50	>500	3.12	500	1.56	1.95
13	50	>500	3.12	500	1.56	1.95
14	100	>500	6.25	500	1.56	1.95
15	>100	>500	3.12	125	1.56	1.95
16	>100	>500	6.25	500	1.56	1.95
17	>100	500	3.12	125	1.56	1.95

SUMMARY AND CONCLUSIONS

Using phenethicillin discs containing either 2, 5, or 10 μg and a standardized technique with an inoculum of moderate size, one can differentiate three categories of sensitivity of staphylococci to three different penicillins by measurement of the size of the zone of inhibition. The largest zones of inhibition occurred with nonpenicillinase-producing organisms, always designated sensitive. Staphylococci producing the smallest zones are penicillinase producers and when very large inocula are used in the tube-dilution method they show great increase in minimal inhibitory concentration with phenethicillin and penicillin G but not with Staphcillin. Such strains are usually reported resistant to penicillin G. The intermediate strains, which are in a separate category not accepted by all observers, produce zones distinctly smaller than those of the sensitive but larger than those of the resistant organisms. With phenethicillin the minimum inhibitory concentration is not elevated when a large inoculum is used.

The above findings indicate that single phenethicillin discs, preferably the 2- μg disc, can be used to differentiate the degrees of sensitivity of staphylococci to all penicillins. This information would be most useful to the physician if clinical correlation with these laboratory results is achieved. This entails showing that sensitive organisms are treatable by any of the three penicillins tested. The intermediate organisms should be amenable to therapy with phenethicillin and 2,6-dimethoxyphenylpenicillin, or possibly with very high doses of penicillin G [10], whereas the resistant organisms are treatable only with 2,6-dimethoxyphenylpenicillin. This drug has been shown to have a therapeutic effect on staphylococci of all categories of resistance to penicillin G. It remains to be proven that clinically successful phenethicillin treatment of moderately resistant but not resistant organisms as outlined by the laboratory tests is possible.

The results of these experiments indicate that on present standards phenethicillin discs are more reliable than penicillin-G discs for outlining the degrees of resistance of staphylococci to the penicillins. Bauer [11], using another method and interpretation, has achieved good results with penicillin-G discs in outlining the three categories of staphylococcal sensitivity to penicillin G.

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A COMPARISON OF THE BIOCHEMICAL ACTIVITIES OF 6-AMINOPENICILLANIC ACID, BENZYLPENICILLIN, 2,6-DIMETHOXYPHENYLPENICILLIN, α-PHENOXYETHYLPENICILLIN, AND PHENOXYMETHYLPENICILLIN

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INTRODUCTION

As an approach to the problem of the correlation of chemical structure with activity a number of penicillins including 6-aminopenicillanic acid, benzylpenicillin, 2,6-dimethoxyphenylpenicillin, phenoxymethylpenicillin, and two stereoisomers of α-phenoxyethylpenicillin were compared with respect to some of their biochemical and biological activities. This paper reports on relative reactivities with penicillinase from Bacillus cereus and Staphylococcus aureus, growth-inhibition of penicillinase-producing strains of B. cereus and S. aureus, and induced formation of penicillinase in B. cereus and S. aureus.

MATERIALS AND METHODS

6-Aminopenicillanic acid (6-APA) was kindly supplied by Chas. Pfizer and Co., benzylpenicillin (penicillin G) by E. R. Squibb and Sons, phenoxymethylpenicillin (penicillin V) by Eli Lilly and Co., and 2,6-dimethoxyphenylpenicillin* and the D- and L-diastereoisomers of α-phenoxyethylpenicillin† by Bristol Laboratories. The test organisms were B. cereus (ATCC 10876; NRRL B-569) and a clinically isolated strain of S. aureus [1]. The enzymes used were cell-free preparations of penicillinase obtained from B. cereus or S. aureus which were partially purified and concentrated by isoelectric precipitation [2].

Minimum growth-inhibitory concentrations of the several penicillins were determined by turbidimetric measurements of the rate of growth of cultures in trypticase soy broth over a 5-hr period.

Rates of enzymatic hydrolysis of the penicillins were measured manometrically in the Warburg apparatus through liberation of CO_2 from a $NaHCO_3$ buffer system by formation of penicilloic acid [3]. Induced formation of penicillinase by the various penicillins in B. cereus and S. aureus were carried out as described in a previous paper [1].

RESULTS

Inhibition of growth of B. cereus and S. aureus. Inhibitory concentrations of the several penicillins were determined by their effects on the rate of growth. The minimal concentration of each antibiotic which prevented any significant increase in the turbidity of shake cultures in a 5-hr period is given in Table 1. The phenoxymethyl and the two phenoxyethyl isomers showed relatively minor but interesting

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*The trade name of Bristol Laboratories for 2,6-dimethoxyphenylpenicillin is Staphcillin.

†The trade name of Bristol Laboratories for the DL racemic mixture of α-phenoxyethylpenicillin is Syncillin.

Table 1. Growth-Inhibiting Concentrations, μg/ml, at High Initial Cell Concentrations

Penicillin	*B. cereus*	*S. aureus*
Benzylpenicillin	20	8000
Phenoxymethylpenicillin	40	8000
D-α-Phenoxyethylpenicillin	100	4000
L-α-Phenoxyethylpenicillin	40	6000
2,6-Dimethoxyphenylpenicillin	20	2
6-Aminopenicillanic acid	2000	400

Turbidimetric measurements were made at periodic intervals of shaken cultures of the organisms in a series of concentrations of the several penicillins. Overnight cultures of the test organisms were diluted to yield an initial concentration of 10^8 organisms/ml. Despite the relatively high inocula, growth was exponential under the experimental conditions. The minimum concentration of compound which prevented any significant increase in the turbidity of the culture in 5 hr was considered to be the growth-inhibiting concentration.

differences from benzylpenicillin. D-α-phenoxyethylpenicillin was slightly more effective against *S. aureus* than the L-isomer. However, against *B. cereus* the reverse was true. The effect of 2,6-dimethoxyphenylpenicillin was much more striking. It was no more active against *B. cereus* than benzylpenicillin. Against *S. aureus*, however, 2,6-dimethoxyphenylpenicillin was more than 1000 times more effective than benzylpenicillin. 6-aminopenicillanic acid was rather ineffective against *B. cereus* but showed a surprising degree of activity against *S. aureus*.

Relative susceptibility to destruction by penicillinases. Phenoxymethylpenicillin was hydrolyzed at a greater rate by the penicillinases derived from both *B. cereus* and *S. aureus* than was benzylpenicillin (Table 2). The two isomers of α-phenoxyethylpenicillin again displayed interesting differences. The L-isomer was very rapidly destroyed by staphylococcal penicillinase at a rate three times greater than that of the D-isomer. In this situation the relative reactivities were reversed when tested with the enzyme produced by *B. cereus*. In contrast to the above compounds, 2,6-dimethoxyphenylpenicillin was hydrolyzed at very low rates relative to benzylpenicillin. It was somewhat more resistant to the penicillinase

Table 2. Rates of Enzymatic Hydrolysis of Several Penicillins Relative to Benzylpenicillin

Penicillin	Source of penicillinase	
	B. cereus	*S. aureus*
Benzylpenicillin	100	100
Phenoxymethylpenicillin	120	125
D-α-Phenoxyethylpenicillin	140	60
L-α-Phenoxyethylpenicillin	80	200
2,6-Dimethoxyphenylpenicillin	1.6	0.35
6-Aminopenicillanic acid	0.8	0.10

Rates of hydrolysis of the penicillin at 0.01 M concentrations were measured manometrically and calculated from initial linear portions of the curves for CO_2 release. The penicillinase solutions were cell-free preparations assaying approximately 10,000 units (μmoles penicillin G hydrolyzed/hr) per ml. They were used as such for the slowly reacting penicillins and at a 1:100 dilution for the rapidly reacting penicillins.

Table 3. Relative Rates of Hydrolysis of Mixtures of Isomers of α-Phenoxyethylpenicillin by Staphylococcal Penicillinase

Molar concentrations of mixtures $\times 10^{-3}$		Relative rate of hydrolysis of mixture, (benzylpenicillin=100)
L-isomer	D-isomer	
3.3	0	180
3.3	0.33	115
3.3	1.65	75
3.3	3.3	67
0	3.3	60

produced by S. aureus than to the enzyme produced by B. cereus. 6-Aminopenicillanic acid was attacked at one third to one half the rates of 2,6 dimethoxyphenylpenicillin by the respective penicillinases.

On comparing a commercial preparation of racemic DL-α-phenoxyethyl) penicillin* with the D- and L- isomers it was found that the mixture was hydrolyzed by both B. cereus penicillinase and S. aureus penicillinase at slower rates than was benzylpenicillin (90% and 75%, respectively). Thus, with the B. cereus enzyme the racemic mixture was hydrolyzed at a rate approaching that of the slower acting L-isomer and with the S. aureus enzyme the mixture was hydrolyzed at the

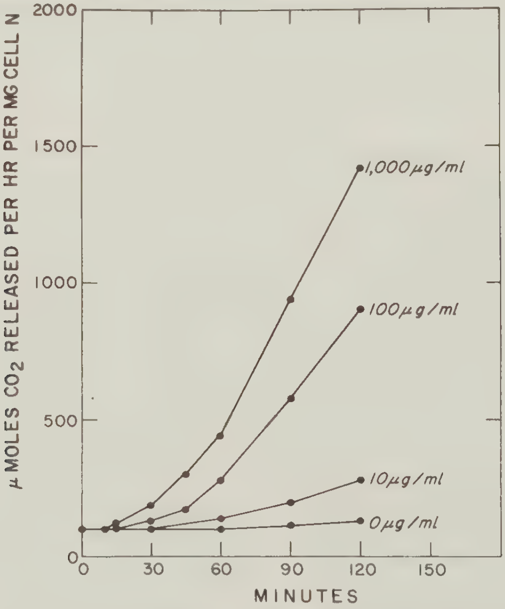

Fig. 1. The induced formation of penicillinase in S. aureus in response to a series of concentrations of benzylpenicillin. Washed cells from an overnight trypticase soy broth culture were added to yield an initial concentration of 50 μg (dry weight)/ml. Induction was carried out in trypticase soy broth containing from 10 to 1,000 μg penicillin G/ml in an incubated shaker at 37 C. Samples were removed from a 20-ml reaction mixture at periodic intervals and the induction reaction was halted by inactivation of the organisms with 10^{-3} M p-chloromercuribenzoate. The penicillinase content thus stabilized was determined manometrically. The resultant penicillinase activities, corrected for growth of the organism during the course of the reaction, were plotted against the time of withdrawal of the sample.

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*Such preparations usually contain a higher percentage than the expected 50% of the L-isomer [4].

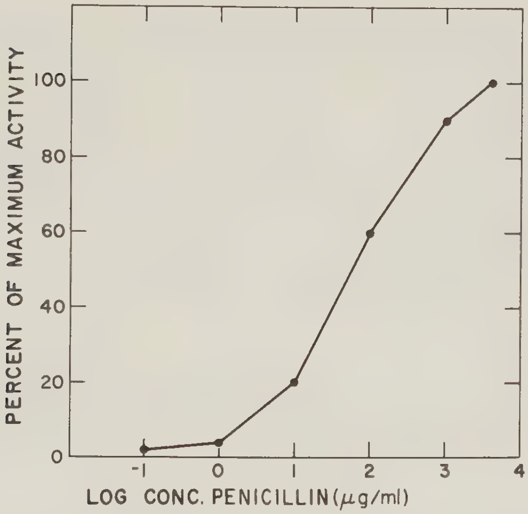

Fig. 2. The effect of the inducing concentration of benzylpenicillin on the rate of enzyme formation by S. aureus in broth medium. The experimental conditions were those described in the legend of Fig. 1. The increase over the basal rate of the specific activities (μmoles of penicillin G hydrolyzed/hr/mg cell N) of the penicillinase obtained after an induction period of 120 min with a series of concentrations of benzylpenicillin are compared relative to the maximum activity achieved with 4000 μg/ml.

rate of the less reactive D-isomer (Table 2). The interaction of two isomers, presumably responsible for these results, was investigated directly by adding various concentrations of the D-isomer to a fixed concentration of the L-isomer. As shown in Table 3, increased ratios of the D-isomer caused greater inhibition of the rate of hydrolysis of the L-isomer by staphylococcal penicillinase until, at a 1 : 1 ratio, the resultant rate was that of the D-isomer alone.

Induction of penicillinase in B. cereus and in S. aureus. Penicillinase-producing cultures of S. aureus are stimulated to elaborate additional penicillinase by the presence of benzylpenicillin [1]. The rates of enzyme formation in S. aureus resulting from several inducing concentrations of benzylpenicillin are shown in Fig. 1. It is evident that the rate of production of the enzyme was dependent on the initial concentration of the penicillin.

Figure 2 shows the relative amount of enzyme formed by S. aureus as a function of the inducing concentration of benzylpenicillin. The concentration required to achieve half the maximum rate of enzyme formation, derived from this curve, is considered as the "induction constant" [5]. Table 4 summarizes the "induction constants" with both B. cereus and S. aureus for the several penicillins. Except for 6-aminopenicillanic acid the values for B. cereus were very similar. 2,6-Dimethoxyphenylpenicillin was almost equally as effective in inducing enzyme formation in S. aureus as in B. cereus. The other substituted penicillins, however, were active only at concentrations 100 times higher than with B. cereus. 6-Aminopenicillanic acid, by this criterion, was somewhat more effective with S. aureus than with B. cereus.

Table 5 summarizes the results on the increase in the amount of penicillinase obtained with optimal concentrations of each inducer. It is evident that 2,6-dimethoxyphenylpenicillin was a very good inducer for both B. cereus and S. aureus. The other compounds were much less effective for S. aureus than B. cereus. Poor induction systems were characterized not only by smaller amounts of enzyme formed but also by higher inducing concentrations required (Table 4).

Table 4. Inducing Concentrations, mg/ml, Forming One Half the Maximum Amount of Penicillinase Obtained After a Specific Induction Period

Inducer	B. cereus	S. aureus
Benzylpenicillin	0.05	40
Phenoxymethylpenicillin	0.04	40
D-α-Phenoxyethylpenicillin	0.04	10
L-α-Phenoxyethylpenicillin	0.10	10
2,6-Dimethoxyphenylpenicillin	0.05	0.5
6-Aminopenicillanic acid	20	2

The amounts of enzyme formed after 120 min were plotted as a function of the concentration of the inducer. The concentrations required to achieve one half the maximum amounts of enzyme obtained with optimal concentrations of the respective inducers were derived from such plots.

Table 5. Ratio of Induced Penicillinase Activity to Basal Activity After a Specific Induction Period

Inducer	B. cereus	S. aureus
Benzylpenicillin	500	40
Phenoxymethylpenicillin	450	37
D-α-Phenoxyethylpenicillin	400	43
L-α-Phenoxyethylpenicillin	480	48
2,6-Dimethoxyphenylpenicillin	800	300
6-Aminopenicillanic acid	100	20

Total penicillinase activity (units/mg cell N) resulting from 120 min induction with optimal concentrations of each inducer were compared relative to the basal activity. The penicillins were added when logarithmically growing broth cultures reached a standard optical density corresponding to 10^8 cells/ml.

DISCUSSION

The present report describes studies in which several new penicillins have been compared with respect to certain properties of benzylpenicillin and phenoxymethylpenicillin, some of whose biological activities have been examined previously [5]. The D- and L-diastereoisomers of α-phenoxyethylpenicillin, homologs of phenoxymethylpenicillin, possessed somewhat greater differences in reactivities from benzylpenicillin than did phenoxymethylpenicillin, which, other than its higher reactivity with the penicillinases of B. cereus and S. aureus, closely resembled benzylpenicillin in its biochemical properties. In addition to the effect of the additional radical in the side chain of the penicillin molecule, the two isomers showed numerous stereo-specific differences. The L-isomer was destroyed much more rapidly by staphylococcal penicillinase than was the D-isomer and was also slightly less effective in inhibiting growth of S. aureus. On the other hand the D-isomer was attacked more readily by the penicillinase of B. cereus than was the L-isomer and in this instance also the penicillinase-resistant isomer was the more effective antibiotic. It must be noted, however, that penicillinase-resistance is not the only factor governing the biologic activity of a penicillin. L-α-phenoxyethylpenicillin, which was hydrolyzed by the penicillinase of S. aureus at twice the rate that benzylpenicillin was hydrolyzed, was, nevertheless, slightly more effective against growth of the organism under the test conditions.

Taking into account the effect of inoculum size of the penicillinase-producing Staphylococcus on the inhibiting concentration of a penicillinase-susceptible penicillin, the results on the specificity of the isomers of α-phenoxyethylpenicillin and on the interaction of mixtures of the isomers are in accord with the data in the literature [4,6,7]. The interaction of the two forms appears to be due, at least in the staphylococcal system, to inhibition of the L-isomer by the D-form. The situation here does not appear to be identical with that of cycloserine where two stereoisomers, each with biological activity, are thought to function by independent mechanisms [8].

Lesser differences between the two stereoisomers of α-phenoxyethylpenicillin were evident in the penicillinase induction reactions in B. cereus and S. aureus when the enzyme was induced with a specific penicillin and the resultant penicillinase was measured separately with benzylpenicillin as the substrate. However, when the L-isomer was both inducer and substrate in a single reaction system with S. aureus, liberation of CO_2 proceeded more rapidly than with either the D-isomer or with benzylpenicillin. Although this effect might be construed as a greater stimulation of enzyme production by the L-isomer it is actually the result of the greater rate of destruction of the substrate.

The efficacy of 2,6-dimethoxyphenylpenicillin with S. aureus in both the growth-inhibiting and the induction reactions, as compared with benzylpenicillin, is presumably a result of its persistence due to its relative insensitivity to attack by penicillinase. That there was no corresponding difference between the two compounds in the inhibition of growth of B. cereus may be a reflection of the much lower basal content of penicillinase in the latter organism. In contrast to the situation with S. aureus, 2,6-dimethoxyphenylpenicillin and benzylpenicillin were equally effective in inducing penicillinase in B. cereus. A possible explanation of this striking difference is that the inducing complex postulated by Pollock [9] may not be as immune to the destructive action of penicillinase in S. aureus as it appears to be in B. cereus.

A number of studies similar to the present investigation have been made on other penicillins [4, 5, 10, 11, 12]. Although many interesting correlations have emerged from these investigations only a beginning has been made in this field. When the many new penicillins now being fashioned [13] become available for comparative studies, more precise and comprehensive correlation of chemical structure with biologic and biochemical activity will undoubtedly result.

ACKNOWLEDGMENT

The technical assistance of Miss Emmy Lou Denny is gratefully acknowledged.

SUMMARY

Other than its greater reactivity with the penicillinases derived from Bacillus cereus and Staphylococcus aureus, phenoxymethylpenicillin closely resembled benzylpenicillin in the minimum growth-inhibiting concentrations against the two organisms and the concentrations required to induce penicillinase in the two organisms.

Two diastereoisomers of α-phenoxyethylpenicillin showed numerous differences from benzylpenicillin and from each other. The D-isomer was hydrolyzed

more rapidly than the L-isomer by the penicillinase of *B. cereus* and was less effective in inhibiting growth of the organism. The converse situation occurred with *S. aureus*. The L-isomer was more rapidly destroyed by the enzyme produced by the latter organism and was of lower biologic activity. The two isomers showed a strong antagonistic interaction in that the D-isomer competitively inhibited the action of the staphylococcal enzyme on the L-isomer. However, in the induction reactions with *B. cereus* and *S. aureus* the two isomers were similar to each other and to benzylpenicillin.

Although characterized by a certain degree of specificity, the rates of hydrolysis of the phenoxymethyl and phenoxyethyl compounds varied within a limited range from the corresponding rates of benzylpenicillin. In marked contrast 6-aminopenicillanic acid and 2,6-dimethoxyphenylpenicillin were hydrolyzed by the penicillinase of *B. cereus* at very low rates and by the staphylococcal enzyme at still lower rates. 6-Aminopenicillanic acid, which was a weak growth-inhibitor against *B. cereus*, possessed some activity against *S. aureus*. 2,6-Dimethoxyphenylpenicillin, which was as effective as benzylpenicillin against *B. cereus*, was greatly superior to benzylpenicillin against high concentrations of *S. aureus*. Benzylpenicillin and 6-aminopenicillanic acid were considerably less active as inducers in *S. aureus* than in *B. cereus*. 2,6-Dimethoxyphenylpenicillin, however, was equally effective with both organisms.

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DISCUSSION

DR. WISE (Food and Drug Administration): Would you please indicate the concentrations of penicillin and penicillinase in the slide showing the reaction rate of penicillinase upon 2,6-dimethoxyphenylpenicillin. Did a change in concentration in either direction yield different results?

DR. STEINMAN: The penicillins are used at 0.01M concentrations. The enzyme solutions were concentrated preparations containing about 10.000 units/ml. The unit we use is μmole penicillin G destroyed per hr/mg of cell N. For the unreactive compound, dimethoxyphenylpenicillin, the rate of hydrolysis was so low that we could not measure it with conventional amounts of penicillinase. Hence, we used 100 times concentrated enzyme preparations. We need more sensitive methods of assay to detect the very low rates of destruction of the antibiotic that occur with less active enzyme preparations or with lower substrate concentrations. The question of concentration is very important. Dimethoxyphenylpenicillin is a very poor substrate for the enzyme not only as to relative rates of reaction but also with respect to affinity for the enzyme, i.e., it has a very high K_m. Penicillin G is hydrolyzed at a constant rate until a low level is reached. In contrast, the rate of hydrolysis of dimethoxyphenylpenicillin, even when starting at a 0.01 M concentration, falls continuously.

DR. SAZ: I wonder what validity you give to the value of 0.35 for 2,6-dimethoxyphenylpenicillin for hydrolysis relative to penicillin G at 100. Is it not possible that even with this low rate of hydrolysis of dimethoxyphenylpenicillin coupled with the high rate of induction of penicillinase you might not get destruction of the dimethoxyphenylpenicillin. If this is so, why is the organism inhibited? Could dimethoxyphenylpenicillin act in a manner different from that of penicillin G?

DR. STEINMAN: That is a crucial question. Penicillinase happens to be a very powerful enzyme and even a low rate of hydrolysis would result in the destruction of a great many molecules of penicillin per minute. In fact, if we increase the enzyme concentration we can completely destroy the dimethoxyphenylpenicillin but, as has been pointed out, this is possible only with a hundredfold higher enzyme level than for penicillin G. As I have indicated in the discussion on the relative affinities of the substrates for the enzyme, we have in the high K_m of the compound one reason why dimethoxyphenylpenicillin persists a little longer than expected from the initial rate of hydrolysis. Why the dimethoxy-derivative is more effective against S. aureus than penicillin G, particularly in view of the fact that they are equally effective against B. cereus, is a complex problem and probably rests on more than relative resistance to penicillinase.

DR. SAZ: Perhaps I didn't make myself clear enough. I may be all wrong because I don't know anything about Staphcillin, just what I hear. The assumption is that Staphcillin is active against penicillin-resistant staphylococci because Staphcillin is not hydrolyzed. I wonder if Dr. Steinman has calculated whether the amount of penicillinase produced under the impact of Staphcillin is enough to destroy the Staphcillin despite the fact that the Staphcillin is only weakly hydrolyzed by the enzyme. The question is why are the resistant staphylococci inhibited by dimethoxypenicillin, since apparently the 2,6 dimethoxy-compound is the best inducer that you have and even though the rate of hydrolysis is low, do you have these two competing reactions. What I want to know is whether any calculations have been made indicating that theoretically enough dimethoxypenicillin is hydrolyzed by the penicillinase which is induced so that the dimethoxypenicillin should not be effective against resistant staph.

DR. STEINMAN: The role of induction of penicillinase is difficult to assess in the in vitro situation. Although an independent reaction, it is a function of the concentration of the inducer and of the persistence of the inducing penicillin-organizer complex. It is certainly true that the superior inducing ability of the dimethoxy-compound in S. aureus should cause this drug to be less effective than if induction were not to occur. Nevertheless, despite the finite destruction of the compound and the adverse formation of induced penicillinase, the data show that dimethoxyphenylpenicillin is effective against penicillinase-producing staphylococci.

A word on the role of penicillinase. At this time our evidence is indirect. The well-known phenomenon of inoculum size with respect to MIC of penicillin G against penicillinase-producing S. aureus, wherein the concentration of penicillin required to inhibit growth of the organism rises rapidly with increased initial numbers of cells, would appear to support the thesis that the enzyme plays a major role in resistance.

DR. SAZ: Our interpretation is completely at variance with this because if staph penicillinase is intracellular, and I think that most people agree that it is, the only argument is whether it is inducible or noninducible. If it is an intracellular enzyme, I don't see what the size of the inoculum is going to have to do with the rate of killing. If it's intracellular, even if you have one single organism, there would theoretically be enough penicillinase produced there to destroy the penicillin absorbed. I know that since 1947 this type of data has been reported and so interpreted but still I disagree with this interpretation, because I can't understand how you can explain, if you assume that staph penicillinase is intracellular, how increasing the size of inoculum effectively increases the amount of penicillinase that is present. The amount of penicillinase is fixed within the boundaries of the cell. I don't understand this at all.

DR. STEINMAN: I have one last point to make on this subject. I concur with Dr. Saz that if the penicillinase content of the cell were the only parameter involved in resistance then the initial number of cells in an environment should be of minor importance. This is indeed true for penicillin G acting on penicillin-sensitive strains of S. aureus. However, when dealing with the action of penicillin G on a penicillinase-producing organism, there must be other rate-governing reactions involved which modify the biologic activity of the compound. With the dimethoxyphenylpenicillin, which is relatively insensitive to the destructive action of penicillinase, the phenomenon of effect of inoculum size does not occur. Not only is the MIC remarkably similar (2 μg/ml) for both penicillin-resistant and penicillin-sensitive staphylococci but it is also relatively constant for both very large and very small inocula.

SYNERGISM BETWEEN THE DIASTEREOISOMERS OF α-PHENOXYETHYLPENICILLIN ON SENSITIVE AND RESISTANT STAPHYLOCOCCI

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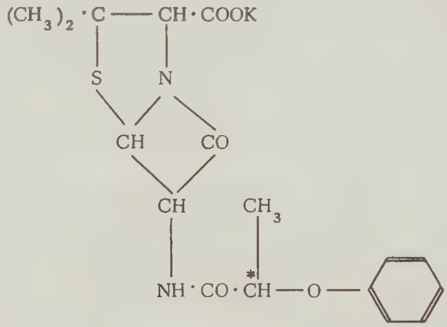
INTRODUCTION

The side-chain of the molecule of α-phenoxyethylpenicillin (Synonyms: α-methylphenoxymethylpenicillin, α-phenoxypropionamidopenicillanic acid) contains an asymmetric carbon (marked by an asterisk in the formula). This compound, therefore, is a mixture of the two diastereoisomeric forms.



* K-α-methylphenoxymethylpenicillin (abbreviated in the text—K-MV)

Perron et al. [1] were the first to prepare both diastereoisomers in pure form and reported that K-D-α-phenoxyethylpenicillin [K-MV(D)] decomposed at 230 to 231 C, $[\alpha]_D^{24} = +251°$. The analytical data for K-L-α-phenoxyethylpenicillin [K-MV(L)] were decomposition at 238 to 239 C, $[\alpha]_D^{24} = +218°$. Both diastereoisomers showed almost analogous infrared absorption spectra in the functional group region whereas location and intensity of the bands in the "fingerprint" region were quite distinctive.

Kraushaar [2] recently reported extensively on the pharmacological and microbiological properties of K-MV, but was, at the time, unable to deal with the properties of the diastereoisomeric components of the mixture. Gourevitch et al. [3], reporting on such investigations, stated that K-MV(D) was less effective than K-MV(L). The mixture of both, however, showed at least the same activity as K-MV(L) and proved to be even more effective for some strains. Apparently we have to deal here with conditions similar to those reported for cycloserine by Smrt et al. [4] and Trivellato [5]. Ciak and Hahn [6a,b], based on these and their own experiments, concluded that the stereoisomers of cycloserine and oxamycin have a different mode of action.

We were therefore led to assume that both diastereoisomers of K-MV show a synergistic interaction and to verify such a probable synergism by quantitative experiments. The aim of our investigations was to establish not only the general principles of such an interaction but to evaluate the activity of both isomers against a series of gradually different sensitive strains of staphylococci. These experiments were prompted by preliminary experiments which showed that one isomer was not always superior to the other since clear reversals of activity occurred. Furthermore, it seemed to be of interest to clarify whether the results of tube tests could be confirmed by a more subtile arrangement, i.e., the examination of the oxygen consumption of bacteria in the Warburg apparatus.

The evaluation of our experiments was done according to the combination diagrams proposed by Loewe [7,8], which were recently interpreted in principle and by example by Zipf et al. [9-10]. We adhered strictly to the nomenclature outlined by Zipf [10] to avoid further misconceptions of the already existing multiplicity of names and interpretations of isoboles, addition, synergism, etc. The basic purpose of such combination diagrams, namely to determine activity or lack of activity, is clearly fulfilled by the serial tube-dilution test.

METHODS

Minimum inhibitory concentration by the serial tube-dilution technique. Strains freshly isolated from clinical material and laboratory test strains of staphylococci were used. The substances under examination were dissolved in broth (Difco nutrient broth) and diluted. The tubes were then inoculated with 0.10 ml broth culture of the respective strains, incubated for 18 hr at 37 C, and diluted to yield a final concentration of 1:200 of the inoculum. All series were incubated for 24 hr at 37 C, read by the naked eye, and the concentration of the first clear tube recorded as the minimum inhibitory concentration (MIC).

Warburg apparatus experiments. The experiments were performed at 37 C and 75 oscillations/min. Each reaction vessel contained 2.9 ml of medium (Difco nutrient broth) and 0.1 ml of 20% KOH. The substances under investigation were dissolved in broth. For the respective concentrations and time intervals of reading, refer to the graphs. Inoculation was with $4 \cdot 10^7$ cells of the various strains as judged by nephelometric measurement.

The following substances were examined: K-α-methylphenoxymethylpenicillin (K-MV) (Trade name Priospen, Biochemie Ges.m.b.H.); K-D-α-methylphenoxymethylpenicillin [K-MV(D)] $[\alpha]_D^{24} = +243°$; and K-L-α-methylphenoxymethylpenicillin [K-MV(L)] $[\alpha]_D^{24} = +216°$.

RESULTS

Table 1 lists the MIC of 29 strains of staphylococci of varying sensitivity arranged according to increasing MIC of the mixture K-MV. It can be seen that the mixture of both diastereoisomers, K-MV, is at least as effective as the single components and with many strains even proves to be superior. Combination diagrams were made with 19 strains and the results, synergism or addition, are shown in the respective columns. It is emphasized that no antagonism was ever encountered.

When examining the MIC of the strains investigated a particular phenomenon concerning the activity of the respective isomers can be noted. There are strains which are more sensitive to the L-isomer, others which react equally to both isomers, and some upon which the D-isomer clearly shows a superior effect. The L-isomer is clearly superior within the range normally considered to be within therapeutic reach, whereas the D-isomer shows superiority when dealing with strains of higher resistance (Table 2).

Table 1. MIC in μg of K-MV, K-MV(D), and K-MV(L) with Strains of Penicillin-Sensitive and Resistant Staphylococci

Strain	K-MV	K-MV (D)	K-MV (L)	Synergism	Addition
SG 511	0.03	0.045	0.0225	+	
Oxford	0.035	0.10	0.03	−	−
209 P	0.035	0.10	0.03	+	
1411	0.04	0.105	0.035		+
5060	0.05	0.075	0.06	+	
5226	0.06	0.156	0.075	+	
1395	0.09	0.15	0.05	+	
42270	0.10	0.15	0.08	+	
1407	0.5	0.75	0.5	+	
1403	0.5	1.2	1.2	+	
1420	0.5	2.5	2.1	+	
Doneu	0.75	0.18	0.10		+
4472	1.2	0.75	0.70	+	
1423	1.2	2.7	2.7	+	
1398	2.0	10	9	+	
R I	5	60	75		+
1397	5	8	4		+
33966	6.2	60	160	−	−
R III	12.5	60	80	+	
Ri	12.5	160	480	−	−
41665	25	60	120	+	
R II	50	160	500	−	−
30471	62.5	200	400	−	−
2595	100	200	500	−	−
43809	100	200	500	−	−
42379	100	120	400	−	−
43555	100	180	400	−	−
48445	150	125	250	+	
Weiss	312	78	625	−	−

- not done

Table 2. Number and Percentage of Strains of Staphylococci Inhibited by K-MV(D) and K-MV(L) Arranged in Ranges of Sensitivity

MIC, μg/ml	K-MV (L)<K-MV (D)				K-MV (L)=K-MV (D)				K-MV (L)>K-MV(D)			
	L		D		L		D		L		D	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
0.01-0.05	6	21	1	3	–	–	–	–	–	–	–	–
0.06-0.10	4	14	4	14	–	–	–	–	–	–	–	–
0.11-1.0	1	3	6	21	–	–	–	–	–	–	–	–
1.1-10	3	10	3	10	2	7	2	7	–	–	–	–
11-100	–	–	–	–	–	–	–	–	2	7	6	21
101-700	–	–	–	–	–	–	–	–	11	38	7	24
Total	14	48	14	48	2	7	2	7	13	45	13	45

To confirm these results the effect of the D- and L-isomers in various concentrations on several strains of staphylococci was examined by the much more sensitive manometric method.

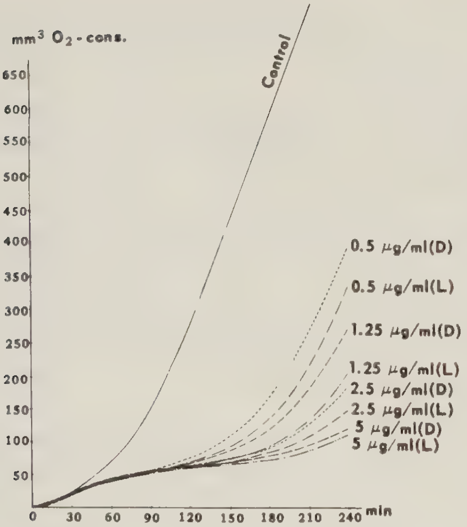


Fig. 1. Effect of various concentrations of K-MV (D) and K-MV(L) on Staphylococcus aureus 1398.

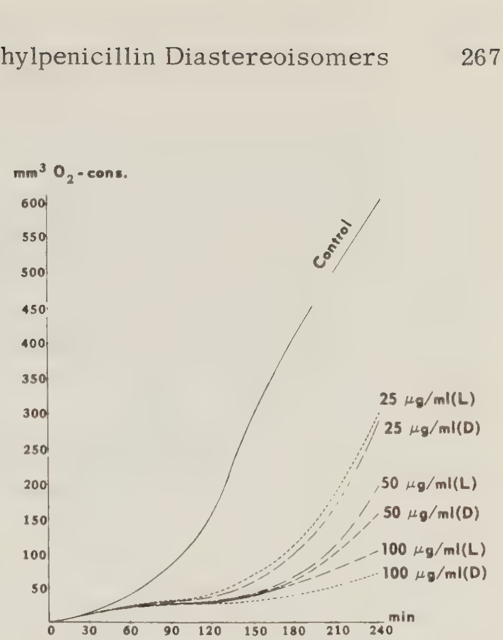


Fig. 2. Effect of various concentrations of K-MV (D) and K-MV(L) on Staphylococcus aureus 48445.

Figure 1 shows that all concentrations of the L-isomer are more effective on the weakly sensitive strain, 1398, the higher the concentrations, the less the differences. The highly resistant strain, 48445, however, reacts oppositely. One can see from Fig. 2 that the D-isomer is the more effective one and that the differences increase with increasing concentrations. These striking observations might be explained by a higher resistance of the D-isomer against penicillinase produced by resistant staphylococci. Penicillinase was produced with strain 30471 according to the method of Ortel [12] and was used to determine the amount required to inactivate 1 mg of the respective isomers. The results were 174 μg for the D-isomer and 120 μg for the L-isomer. There is a certain parallel between resistance and the capacity for penicillin inactivation by penicillinase which might be used as explanation. The relatively small quantitative differences, however, are not considered an adequate explanation.

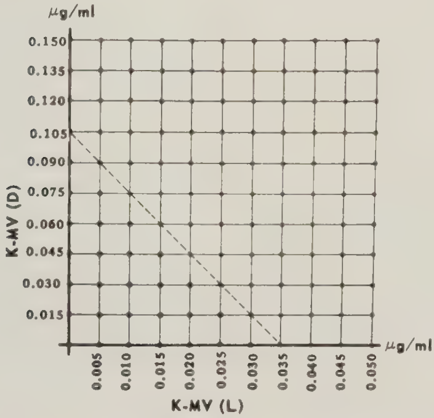


Fig. 3. Combination diagram Staphylococcus aureus 1411.

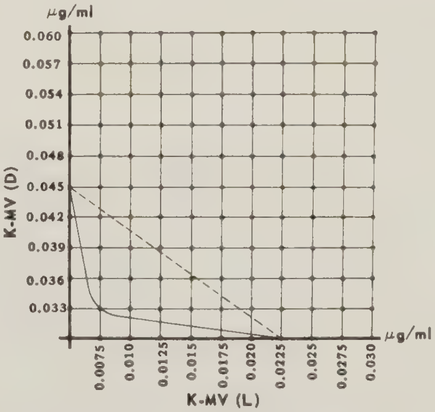


Fig. 4. Combination diagram Staphylococcus aureus SG 511.

Nineteen of the 29 strains under investigation were examined by combination diagrams to detect possible interaction of K-MV(L) and K-MV(D). Figures 3 and 6 demonstrate some of the results obtained.

In the case of Staphylococcus aureus 1411 (Fig. 3), the two isomers show a purely additive interaction. This strain has a normal sensitivity to all kinds of penicillin (G, V, and MV), but was three times more sensitive to the L-isomer of K-MV than to the D-isomer.

Another strain with normal penicillin sensitivity is the laboratory strain, SG 511 (Fig. 4), which shows a definite synergism between the isomers and is more sensitive to the L-form.

Synergism was also exhibited against strain 1398 which is feebly penicillin-sensitive or on the threshold of resistance (Fig. 5), but the MIC of the D- and L-isomers were not strikingly different.

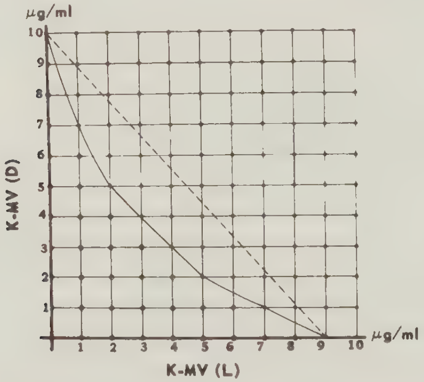


Fig. 5. Combination diagram Staphylococcus aureus 1398.

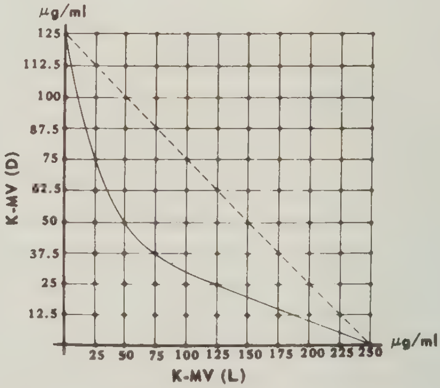


Fig. 6. Combination diagram Staphylococcus aureus 48445.

Strain 48445 is highly resistant and shows synergism when tested in the combination diagram (Fig. 6). Here again, as in earlier tests for MIC, the D-isomer is more effective than the L-isomer, a clear contrast to strains sensitive to penicillin. Synergism was demonstrable at all sensitivity levels, but no regularity of correlation to the degree of sensitivity could be found since an additive interaction was also encountered in all groups. However, it appears that synergism is more pronounced with resistant strains against which the D-isomer is more effective. To reach final conclusions it would be necessary to perform a series of tests with many strains freshly isolated from clinical material.

The combination diagrams represent results of an arbitrarily chosen end point of 18-24 hr. It seemed interesting, therefore, to verify these results by continuous observation, particularly during time intervals of practical clinical importance. It is impossible to perform experiments of such short duration (4 hr) by the serial tube-dilution technique as neither readings by the naked eye nor nephelometric measurements permit any certainty in judging an increase in the bacterial population. It might well be possible to arrive at wrong conclusions when testing a substance effective on bacteria during proliferation only, e.g., orally administered penicillin, because the end point is of no practical importance (therapeutic serum levels, 3-6 hr).

To confirm the combination diagrams by manometric measurements, representative concentrations were selected in preliminary experiments which, by

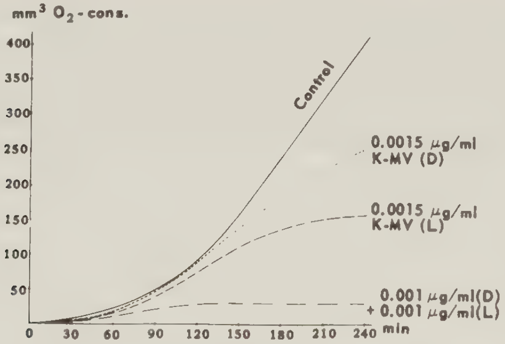


Fig. 7. The effect of K-MV(D), K-MV(L), and their mixtures on Staphylococcus aureus SG 511.

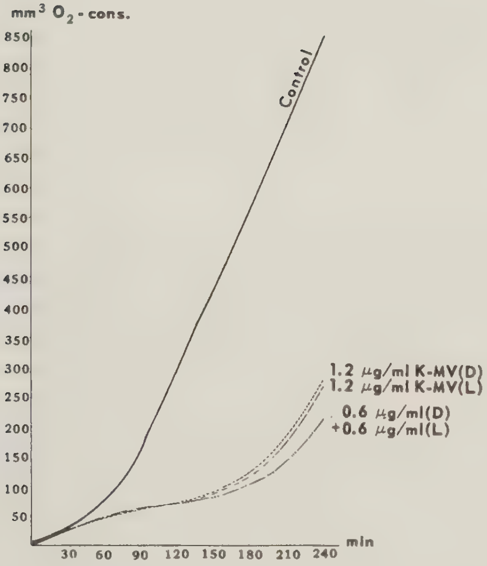


Fig. 8. The effect of K-MV(D), K-MV(L), and their mixture 1 : 1 on Staphylococcus aureus 1398.

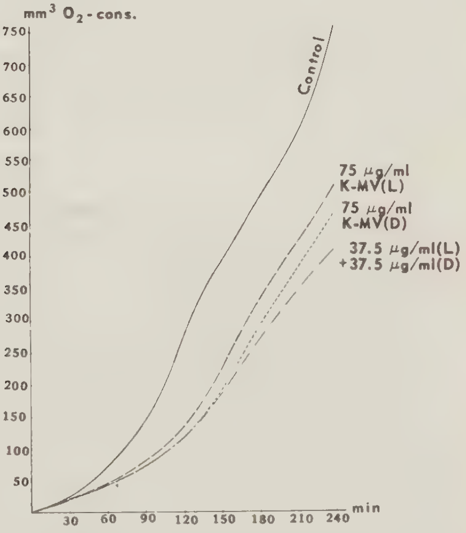


Fig. 9. The effect of K-MV(D), K-MV(L), and their mixture 1 : 1 on Staphylococcus aureus 48445.

simple mixture of both components, promised to show synergism at its best. Figures 7 to 9 show the results of these experiments. Here again the effective quantities of both isomers are easily distinguished for the respective strains.

K-MV(L) was far more effective on strain SG 511 than K-MV(D). The same holds true for strain 1398, although the differences were much smaller, whereas K-MV(D) inhibited resistant strain 48445 much more strongly than did K-MV(L). It was proven in all three cases that the mixtures tested were more effective than the respective single components. These results leave no doubt that a true synergism exists, even with time interval of therapeutic application.

ACKNOWLEDGMENTS

The excellent technical assistance of Miss E. Aspock and Miss G. Kadel is gratefully acknowledged.

SUMMARY AND CONCLUSIONS

α-Methylphenoxyethylpenicillin and its diastereoisomeric forms were investigated for their microbiological properties using strains of staphylococci having varying sensitivity to penicillin. Particular emphasis was laid on the examination of the D- and L-diastereoisomers of this penicillin and their interaction in mixtures. The investigations were performed by serial tube-dilution techniques, combination diagrams, and manometric (Warburg) measurements.

It was found that the L-isomer is more effective than the D-isomer against penicillin-sensitive strains of staphylococci. These differences, however, diminish when strains of lesser sensitivity are used. On the threshold of resistance and with increasingly resistant strains there is a clear cut reversal of activity; for these strains the D-isomer was found to be more effective.

The investigated penicillin, a diastereoisomeric mixture, was found to be more effective than the most effective component. This effect was studied by combining both isomers in well-defined correlation. It was proven beyond doubt that a true synergism exists between the diastereoisomeric fractions, not only for longer lasting (up to 24 hr in in vitro experiments) but also for brief manometric measurements, which correspond to the serum levels of therapeutically administered oral penicillin. Mention is also made of differences concerning the inactivation of the respective diastereoisomers by staphylococcal penicillinase.

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DISCUSSION

DR. DI MARCO (Milano): Please, Dr. Kraushaar, can you tell us something about the mode of action of the isomers of phenoxyethylpenicillin? Have you followed the activity of the two stereoisomers on the lysis of the staphylococcus? Have these two compounds the same lytic activity? In the case of cycloserine, the two different stereoisomers have a completely different mode of action and the synergism that can be deduced from data perhaps must be viewed with certain reservations.

DR. KRAUSHAAR: There is surely different activity against the different strains. I think we have to evaluate a lot of strains to reach a final conclusion. I don't think that the situation is the same as with cycloserine. I think the mode of action would be the same for all penicillins. I would say from this experiment you can't conclude there is a different mode of action.

DR. HAHN: I think in all these instances where one finds additive action proved by the isobologram technique, one has no great difficulties in assuming that additivity is based on identity of action. There is one other possible interpretation which I think would not apply to this situation here. In those instances where synergism is proved the question of differences in action is legitimate. I think the question can only be resolved by chemical studies of the accumulation of cell wall material and the extent to which that accumulation occurs. I don't think that by kinetic studies and growth studies a decision can be arrived at whether one is dealing with identity or difference of mode of action in a state of synergism. I would like to congratulate Dr. Kraushaar for having used these very beautiful and clean-cut criteria for additivity or synergism, because we all are aware that this is a difficult and sometimes controversial subject. I have not been able to understand why this technique of data handling which permits unequivocal conclusions is not more widely used.

ULTRAVIOLET MICROSCOPY OF PSITTACOSIS VIRUS-INFECTED CELLS TREATED WITH ANTIBIOTICS AND WITH ANTIMETABOLITES

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INTRODUCTION

Replication of psittacosis virus (TTS-strain) in cell culture is accompanied by cytochemical changes which reflect nucleic acid biosynthesis [1, 2]. As shown by acridine-orange staining and fluorescence microscopy, DNA-components of the nucleus fluoresce yellow-green and RNA-components of both cytoplasm and nucleolus fluoresce red-orange. In response to infection the host cytoplasm contributed an RNA-staining matrix around the initial DNA-staining viral particle. The resulting inclusion increased in size (1–5 μ) and color intensity (brilliant red), and was distinct from the surrounding RNA-staining cytoplasm. This is referred to as the 14-hr "red ball" stage of virus development (Fig. 1). After 14 hr the cytoplasm was filled with clusters of red-staining, homogeneous, round inclusions. Within 48–72 hr the inclusions proceeded to maturity through a sequence of color changes from shades of red to orange to yellow to green (Fig. 2). At the yellow and green stages (Fig. 3) the viral constituents attained serological maturity and selectively absorbed fluorescein-tagged antibody. This cytochemical sequence of DNA to RNA-matrix to DNA and protein occurred in tissue cultures from which antibiotics had been deleted. The effect of a number of antibiotic and of fluorinated pyrimidine drugs on the cytochemical sequence of replicating virus has been determined.

METHODS

Monolayer cultures of McCoy cells [3] were prepared on coverslips in Leighton tubes. Nutrient medium consisted of Mixture 199 with 0.5% lactalbumin hydrolysate and 2.0% heat-inactivated calf serum. Sufficient psittacosis virus in allantoic fluid was added to each culture so that approximately 95% of the cells were infected. Cultures were incubated at 37 C. At the 14-hr "red ball" stage (Fig. 1) nutrient fluid was exchanged for fresh medium to which graded doses of antibiotics (50 to 0.75 μg/ml of medium) had been added. Noncytotoxic quantities of 5-fluoroorotic acid (FO) and of 5-fluorouracil (FU)* in nutrient fluid were each added to cell cultures showing the 14-hr "red ball" stage of developing virus. The inhibitory effect was further defined by observing the effect of uracil on FU. At daily intervals thereafter coverslip preparations were fixed, stained with acridine orange, and examined by fluorescence microscopy. The technique has been described by Starr et al. [2]. The propagation of psittacosis virus in treated and untreated cultures was determined by inoculation of 10-g mice intracerebrally and by the particle counting method in tissue cultures as described by Tanami et al. [4]. When the mice showed evidence of meningitis, brain imprints of each were stained and examined microscopically for diagnostic inclusion bodies [5].

*Courtesy of Dr. Robert Duschinsky, Hoffmann-LaRoche, Inc., Nutley, New Jersey.

Figs. 1–4. Black and white prints prepared from Ektachrome transparencies in which the effect of ultraviolet light on acridine-orange stained McCoy cells could be observed. In the original preparations RNA-staining components of the cell fluoresced red and DNA-staining components were yellow-green. Viral components are indicated by an arrow. × 1000.

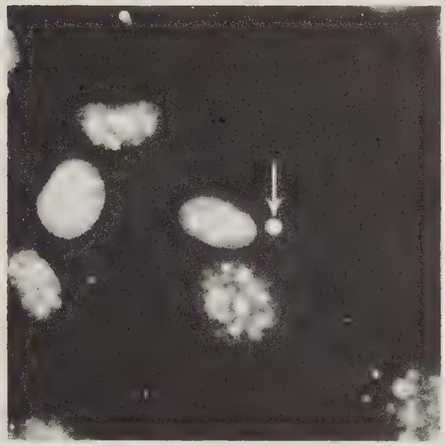

Fig. 1. McCoy cell containing "red ball" stage of replicating psittacosis virus (arrow). The inclusion body appeared red, the cytoplasm and nucleoli red-orange, and nucleoplasm yellow-green.

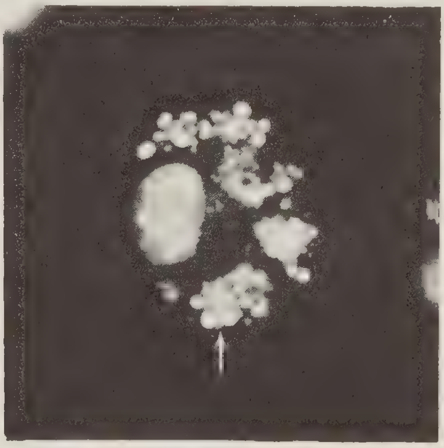

Fig. 2. McCoy cell with maturing psittacosis virus. The round inclusion bodies ranged in color from red to orange to yellow-green.

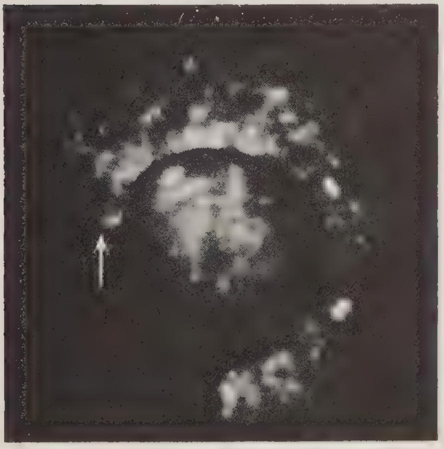

Fig. 3. McCoy cell filled with mature virus particles which fluoresced yellow-green.

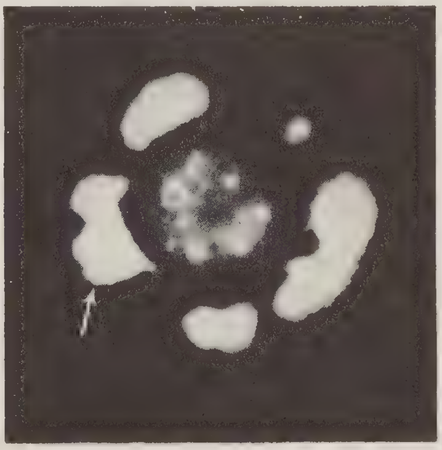

Fig. 4. McCoy cell with "fraudulent" RNA-staining masses in the cytoplasm at 48 hr after the addition of medium plus 5-fluorouracil to the "red ball" stage of developing virus.

RESULTS

A virustatic drug such as chlortetracycline HCL (CTC) prevented maturation of virus beyond the "red ball" stage (Fig. 1). Such tissue-culture preparations were not infective for mice by the intracerebral route and for fresh cells grown in culture [4] (Table 1). A decreasing order of effectiveness in inhibiting the virus maturation process was noted with the following antibiotics: CTC, oxytetracycline, tetracycline, chloramphenicol, erythromycin, kanamycin, penicillin, and streptomycin. Virus suppression was shown with lower dosages of CTC than with oxytetracycline and tetracycline. CTC destroyed cell-free virus and inhibited intracellular mature virus. A dose-response curve showed that duration of treatment was an important factor in determining optimal dosages of each drug (Moore, R. W., in preparation). Replacement of the virustatic medium with antibiotic-free fluids at intervals provided an opportunity for residual live virus to emerge after additional incubation. The remaining drugs showed little effect, even at dosages which approached cytotoxic levels. Streptomycin was negative. In contrast to the virustatic phenomenon shown by CTC, the addition of 25 μg of FU or of FO to the nutrient medium resulted in formation of a "fraudulent" RNA-staining matrix [6, 7] (Fig. 4). Under such circumstances the DNA-staining viral material stimulated formation of a massive, unorganized RNA-staining matrix which did not undergo further biological maturation as would have been reflected by the normal sequence of color changes. Furthermore infective virus was not recovered from FU- and FO-treated cultures. The inhibitory effect was partially reversed by uracil (Table 2). The nucleoli of cells which were treated with such fluorinated pyrimidines were of normal size or enlarged while in cells which were treated with CTC the nucleoli were small and faded.

Table 1. Effects of Antibiotics on Replication of Psittacosis Virus in McCoy Cells

Drug added	μg/ml	Microscopic effect	Virus recovery 4 days after treatment	
			Mouse inoculation*	Particles/ml†
Chlortetracycline	25	Inhibited	0/6	0
Chlortetracycline	1.5	Inhibited	0/6	0
Streptomycin	200	Maturation	6/6	$2 \cdot 10^5$
Control	–	Maturation	6/6	$2 \cdot 10^6$

*mice infected/mice inoculated
†as determined in tissue culture [4].

Table 2. Effect of 5-Fluorouracil on Replication of Psittacosis Virus in McCoy Cells

Drug added	μg/ml	Virus recovered 4 days after treatment	
		Mouse inoculation*	Particles/ml†
5-Fluorouracil	6	0/5	20
5-Fluorouracil+uracil	6+120	2/5	$1.1 \cdot 10^3$
None	–	5/5	$1.1 \cdot 10^7$

*mice infected/mice inoculated
†as determined in tissue culture [4].

DISCUSSION

In response to infection the host cytoplasm contributed an RNA-staining matrix around the initial DNA-staining virus particle. Chlortetracycline blocked continued formation of the RNA-staining matrix and thereby inhibited further maturation of the viral inclusion beyond the 14-hr "red ball" stage at which it had been added. FU and FO did not interfere with RNA formation; however, the fluorinated RNA-staining material thereby formed could not participate in the chemical maturation of the virus. The interference in the RNA biosynthetic sequence has been charted by Harbers et al. [8] as occurring at the level of the polymerization reaction. While viral replication was interrupted by CTC and by the fluorinated antimetabolites, the mechanisms appeared to follow different pathways.

When examined by acridine-orange fluorochrome procedures, the replication pattern of psittacosis virus in tissue culture offers a visual chemical indicator system for study of nucleic acid metabolism, antimetabolities, and for screening of chemotherapeutic agents. It has been applied in a study on trachoma virus propagating in McCoy tissue cells [9], in which chlortetracycline and a macrolide-type antibiotic were virustatic.

ACKNOWLEDGMENT

Supported in part by USPHS grant E-3070 and the James W. McLaughlin Fellowship Fund.

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DISCUSSION

DR. HAHN: I cannot remember having ever seen before such an interesting combination of a number of different techniques and approaches. Would you please explain one technical feature to me? What concentration of acridine orange have you been using in these experiments?

DR. POLLARD: Well, there are some tricks to this acridine-orange technique which have been reported already. We fix the coverslips which have the tissue cultures growing on them in acid alcohol and then adjust the wash to a pH of 3.8. You have to use 3.8, or under 4, for about 4 min and then immerse the coverslips in acridine orange, 0.01% buffered at 3.8, for 4 min. Then we wash the coverslips very thoroughly for another 4 min in pH 3.8 buffer and mount the coverslips in 3.8 buffer. It's as simple as that.

DR. SAZ: Does this have any effect on the tissue cell growth itself?

DR. POLLARD: Oh, yes, that's why they are so valuable in treatment of cancer. We titrated the cytotoxicity first and used a level which was not visibly cytotoxic. These levels interfered, of course, with multiplication of cells but what we were interested in was the use of this chemical indicator system within the cells, the cells acting merely as carrier. But yes, they do interfere with multiplication of cells. We geared concentrations to suit our purpose here.

STUDIES ON THE ANTIMICROBIAL NATURE OF ACTION OF AZASERINE*

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INTRODUCTION

Since the discovery of azaserine (O-diazoacetyl-L-serine) and the detection of its marked antineoplastic and antimicrobial activity, numerous efforts have been made to elucidate the mechanism by which this antibiotic inhibits essential cellular processes [1-7]. Impressive evidence has been collected which indicates that one site of azaserine inhibition in bacteria [8] and mammalian [9,10] cells is the amidination of formylglycinamide ribotide by glutamine, resulting in a blockade of de novo synthesis of purines. Acceptance of this site of inhibition as the sole explanation of the mechanism of action of azaserine has been difficult due primarily to the fact that in microorganisms azaserine inhibition is best prevented by amino acids [11], notably phenylalanine and tyrosine in the case of Escherichia coli [12].

Interpretation of some of the data in the literature is difficult. Much of the antimicrobial work has been done in liquid medium. Azaserine causes extensive filament formation [13] and Kaplan et al. [14] have clearly demonstrated that turbidimetric measurement alone as an index of azaserine inhibition and metabolite reversal in E. coli can lead to false interpretations. To avoid this problem and at the same time use growing cells to verify the growth-essential nature of inhibited and reversed reactions we elected to use the spread-plate technique in a series of studies designed to clarify certain aspects of this problem.

MATERIALS AND METHODS

Microorganisms and conditions. E. coli ATCC 9637 (obtained from Dr. Bernard D. Davis and carried in this laboratory since 1952) as well as three mutant strains of E. coli (also generously supplied this year by Dr. Davis) were grown on the synthetic glucose-salts medium of Anderson [15]. In the case of the metabolically deficient strains, the maintenance medium was supplemented with 100 μg of the required amino acid/ml medium (1-phenylalanine for strain M83-5, 1-isoleucine for strain M9F-21, and 1-tryptophan for strain M19-2). Streptococcus faecalis ATCC 8043 was grown on the medium of Flynn [16] as modified by Hutchison [17]. All these organisms were cultured at 37 C (22-24 hr for E. coli and 26-28 hr for S. faecalis). Saccharomyces cerevisiae ATCC 4098 was grown on the synthetic Bacto-Yeast Morphology Agar [18]. Incubation of this organism was at 28 C for 36 hr.

Inhibition-reversal studies. The spread-plate procedure described previously [19, 20] was employed. This procedure, in essence, consisted of the incorporation of graded doses of antibiotic and, when desired, metabolites in melted agar in petri plates. Inoculation was accomplished by spreading a small volume (usual-

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*This study was supported by the Cancer Chemotherapy National Service Center, National Cancer Institute, under National Institutes of Health Contract Number SA-43-ph-1809.

†Affiliated with the Sloan-Kettering Institute for Cancer Research, New York.

ly 0.05 ml) of a three-times-washed suspension of microorganisms on the hardened agar surface. Following incubation for the specified times isolated colonies (usually 50 to 200) appeared on control plates. Minimal inhibitory concentration of azaserine is defined as the lowest concentration completely preventing the appearance of colonies to the unaided eye. "Reversal" is defined as the development of colonies from all of the cells in the inoculum as a result of the incorporation of a metabolite in the medium containing an otherwise inhibitory concentration of azaserine. These definitions are essentially the same as those of Foster and Pittillo [20] and Stim et al. [21] who have used this technique extensively to demonstrate metabolite reversal of inhibitor action.

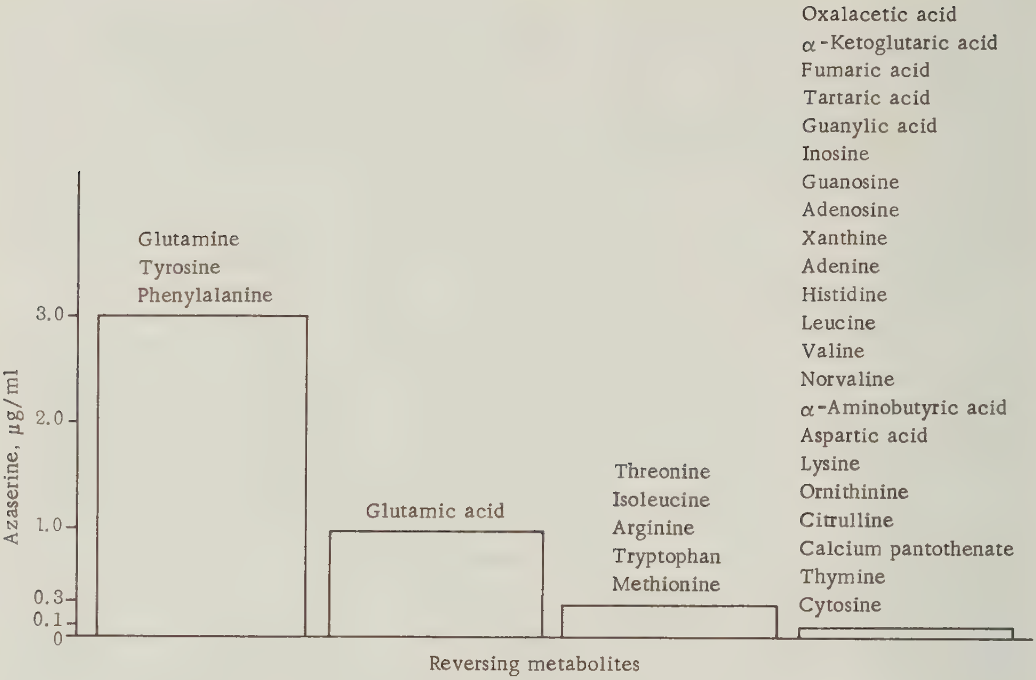
Metabolites tested for azaserine-reversing activity. Initial work consisted of testing the following materials individually for azaserine-reversing activity: glycine, serine, histidine, alanine, threonine, leucine, isoleucine, norleucine, sarcosine, valine, norvaline, α-aminobutyric acid, aspartic acid, glutamic acid, glutamine, lysine, arginine, ornithine, citrulline, cystine, cysteine, methionine, homocysteine, betaine, phenylalanine, tyrosine, tryptophan, proline, hydroxyproline, malonic acid, succinic acid, fumaric acid, citric acid, oxalic acid, formic acid, tartaric acid, α-hydroxy-n-caproic acid, propionic acid, α-hydroxy-n-butyric acid, α-hydroxyisovaleric acid, α-ketoglutaric acid, lactic acid, oxalacetic acid, maleic acid, sodium pyruvate (each tested at a concentration of 100 μg/ml), folic acid, citrovorum factor, ascorbic acid, thiamine, riboflavin, pyridoxal, biotin, nicotinamide, p-aminobenzoic acid, vitamin B_{12}, choline chloride, calcium pantothenate, uracil, thymine, cytosine, adenine, guanine, xanthine, hypoxanthine, adenosine, guanosine, xanthosine, inosine, adenylic acid, and guanylic acid (each tested at a concentration of 10 μg/ml).

RESULTS

Studies on the effect of certain metabolites on the inhibition of microorganisms. The effect of a variety of metabolites on azaserine inhibition of E. coli was determined (Fig. 1). Amino acids, notably glutamine, tyrosine, and phenylalanine, reversed azaserine action. Glutamic acid, threonine, isoleucine, arginine, tryptophan, and methionine demonstrated significant azaserine-reversing activity. Inhibition caused by threshold concentrations of azaserine was reversed by adenine, xanthine, adenosine, guanosine, inosine, guanylic acid, thymine, cytosine, calcium pantothenate, and most α-amino and α-keto acids.

Azaserine inhibition of S. faecalis (see Fig. 2) was reversed most effectively by tyrosine and norleucine. Threshold inhibition by azaserine was overcome by adenine, xanthine, guanylic acid, isoleucine, valine, aspartic acid, glutamic acid, glutamine, lysine, cysteine, cystine, phenylalanine, tryptophan, sodium pyruvate, and fumaric acid. It is of interest that under these test conditions the minimal inhibitory concentration of azaserine for S. faecalis is greater than that for E. coli, 2.0 μg/ml vs. 0.075 μg/ml, respectively. The extent of azaserine reversal is much less in S. faecalis than in E. coli. Tyrosine, for example, reverses 40 times the minimal inhibitory concentration of azaserine for E. coli but only five times the minimal inhibitory concentration of S. faecalis.

Azaserine inhibition of S. cerevisiae was best reversed (Fig. 3) by tyrosine, hydroxyproline, methionine, cysteine, lysine, leucine, threonine, histidine, serine, adenine, and hypoxanthine. Metabolites less effective in reversing azaserine inhibition of this yeast were norleucine, aspartic acid, glutamic acid, arginine, ornithine, citrulline, phenylalanine, proline, sodium pyruvate, cytosine, xanthine, xanthosine, and adenylic acid. An outstanding difference between the



Minimal inhibitory concentration of azaserine for E. coli = 0.75 μg/ml

Fig. 1. The effect of certain metabolites on the inhibition of Escherichia coli by azaserine.

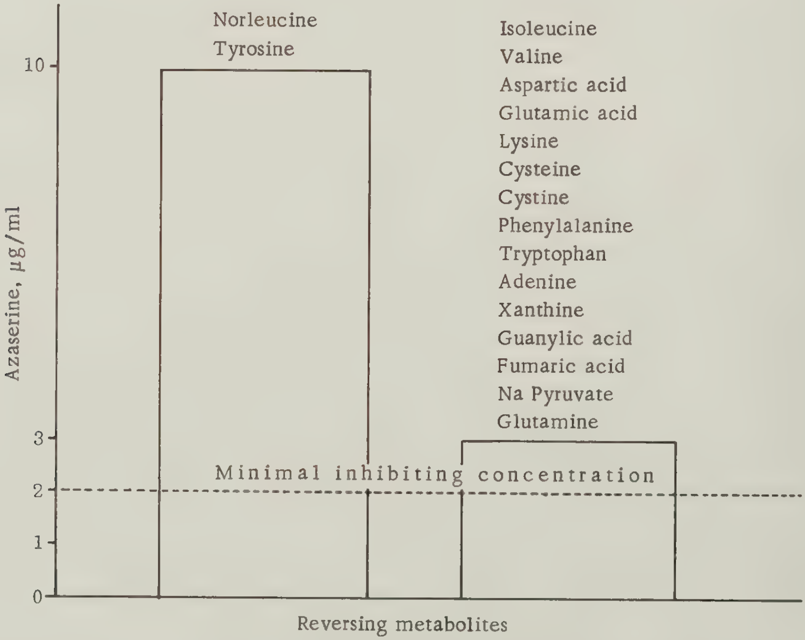


Fig. 2. The effect of metabolites on the inhibition of Streptococcus faecalis by azaserine.

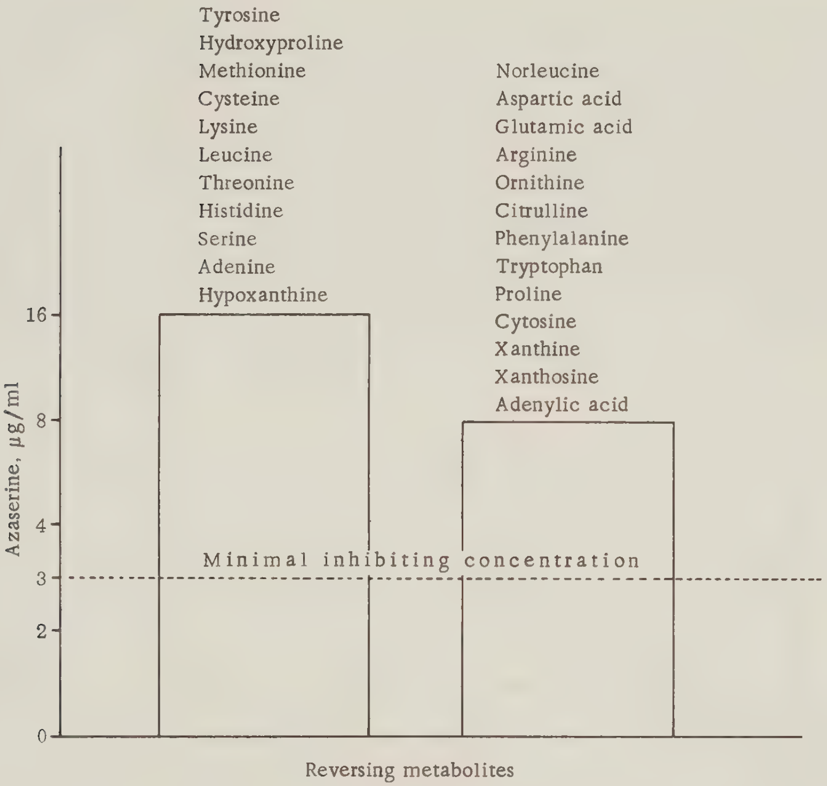


Fig. 3. The effect of metabolites on the inhibition of Saccharomyces cerevisiae by azaserine.

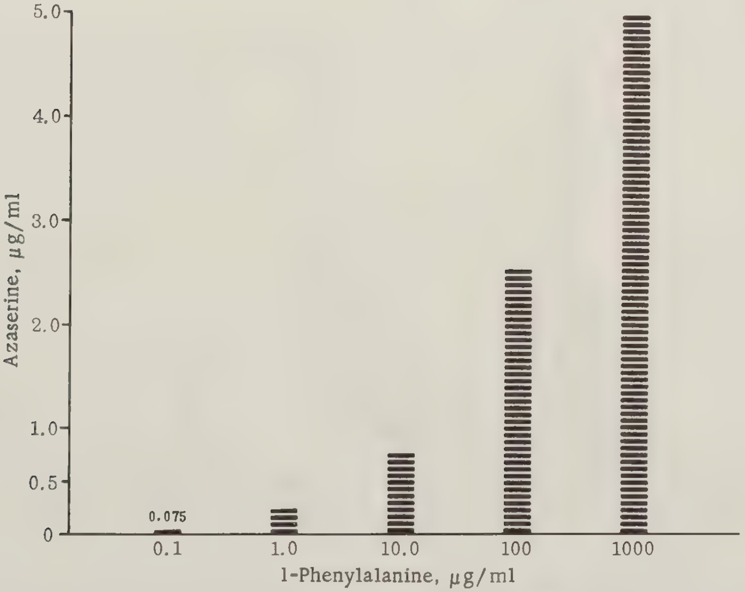


Fig. 4. The effect of l-phenylalanine on the inhibition of Escherichia coli by azaserine.

azaserine-reversal picture in this yeast and in the two bacteria is that in *S. cerevisiae* the purines, adenine and hypoxanthine, appear to be as effective as the most active amino acids in reversing azaserine inhibition.

In *E. coli* the reversing activity of phenylalanine is seemingly competitive for concentrations of azaserine more than 50 times the minimal inhibitory concentration. In the presence of increasing concentrations of phenylalanine, increasing concentrations of azaserine are required for inhibition (Fig. 4). Essentially identical results were obtained with tyrosine and glutamine. Glutamic acid, however, was consistently less active than the other amino acids. On those plates containing azaserine and a concentration of amino acid just adequate to prevent the inhibition caused by the specific concentration of azaserine present, a marked change in the appearance of the colonies was observed. These colonies lacked the moist luster and convex shape of colonies on control plates. Microscopic examination of the atypical colonies revealed that extensive filament formation had occurred.

A similar competitive relationship between azaserine and tyrosine, norleucine, and phenylalanine was shown in *S. faecalis* (Fig. 5). Conclusive evidence was not obtained to indicate a competitive relationship in the case of glutamine and tryptophan. It should be stated that the azaserine-reversing activity of phenylalanine and tyrosine fluctuated in consecutive experiments. Sometimes phenylalanine was as active as tyrosine, other times tyrosine was less active than indicated in Fig. 5. It is our opinion that in *S. faecalis* these two amino acids are probably functioning in an identical manner as far as azaserine reversal is concerned and that some undefined cellular variation from day to day accounts for the apparent differences in reversing activities between these two compounds.

Contrary to previous reports, histidine reversed azaserine inhibition of *E. coli* although it was much less active than phenylalanine. However, histidine in

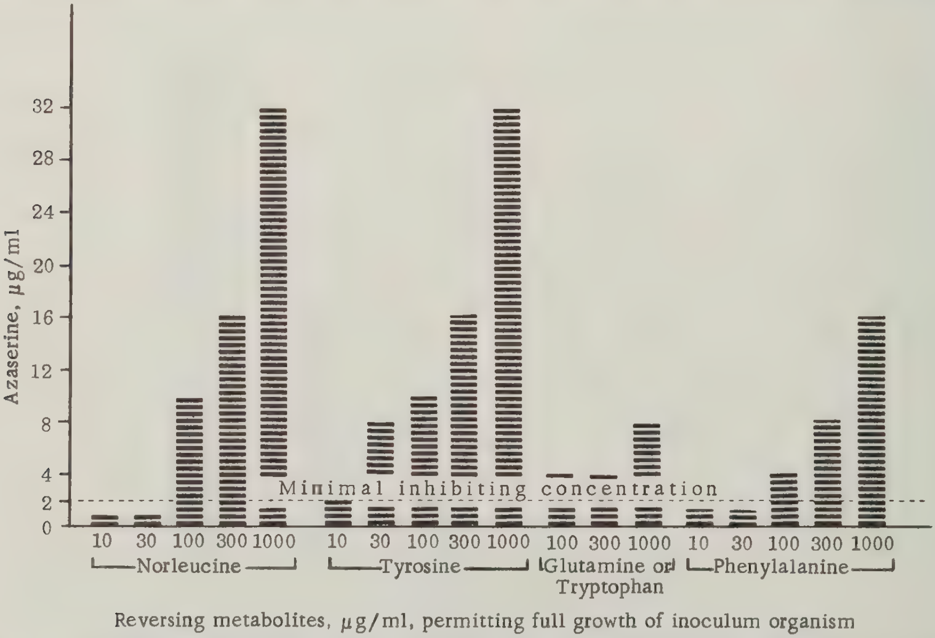


Fig. 5. Concentration relationships between reversing metabolites and azaserine in *Streptococcus faecalis*.

the presence of phenylalanine proved to be a potent azaserine-reversing combination (Fig. 6), confirming earlier observations [14].

The effect of other metabolites on the azaserine-reversing capacity of phenylalanine was determined by supplementing the test medium with 100 μg phenylalanine/ml and increasing the concentrations of azaserine to the point where they were above the reversing action of this concentration of phenylalanine. It was found (Fig. 7) that not only did histidine plus phenylalanine reverse higher levels of azaserine but also phenylalanine plus a number of other individual amino acids, particularly lysine, arginine, ornithine, and norvaline, acted similarly. In the presence of phenylalanine it was observed that adenine, xanthine, adenosine, and inosine were potent reversing agents. Significantly, the reversal of azaserine by phenylalanine was not enhanced by glutamine, a compound which in itself is a potent azaserine-reversing agent.

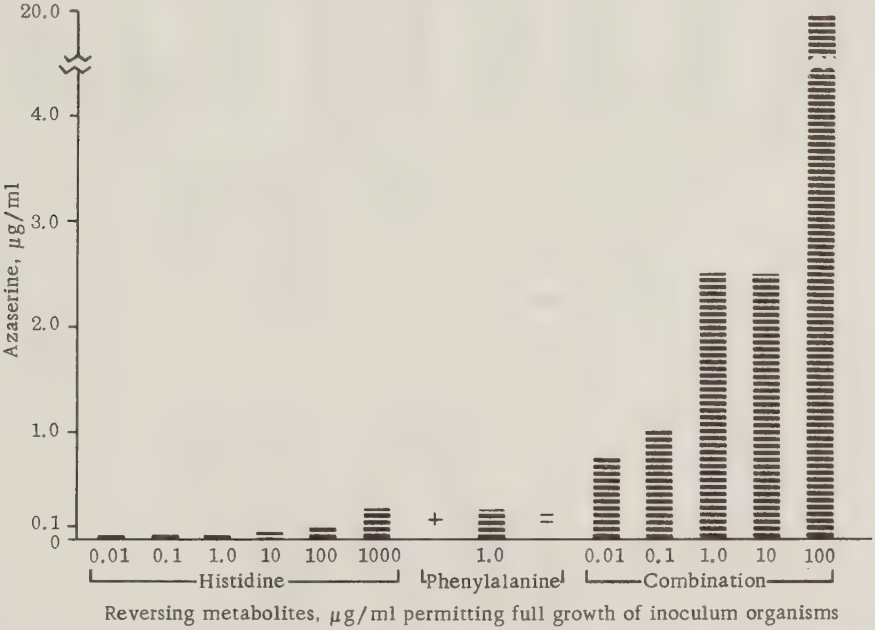


Fig. 6. The effect of histidine and phenylalanine on the inhibition of Escherichia coli by azaserine.

In our initial experiment (Fig. 1) phenylalanine as well as tyrosine, glutamine, and glutamic acid reversed significant concentrations of azaserine and a number of the amino acids as well as some purines had less azaserine-reversing activity. In view of the results obtained when phenylalanine was tested in combination with a variety of materials, it seemed worthwhile to determine the azaserine-reversal pattern of metabolites in the presence of tyrosine or glutamine as well as some of the less potent reversing agents such as adenine, threonine, and α-ketoglutaric acid. This was done in the same manner as with phenylalanine, i.e., by supplementing the medium with the specified metabolite and increasing the concentrations of azaserine to the point where the reversing capacity of the specific concentration of added metabolite was surpassed. All materials were added to the medium in a concentration of 100 μg/ml except the purines, which were tested at 10 μg/ml. It was observed that when the medium was supplemented with glutamine the only metabolites reversing azaserine were

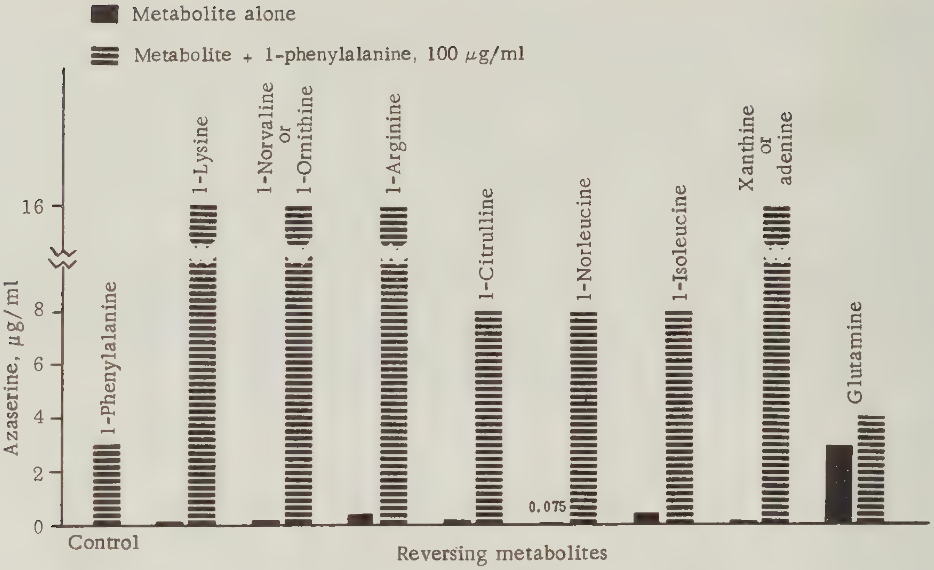


Fig. 7. The effect of certain metabolites on the phenylalanine reversal of azaserine inhibition of Escherichia coli.

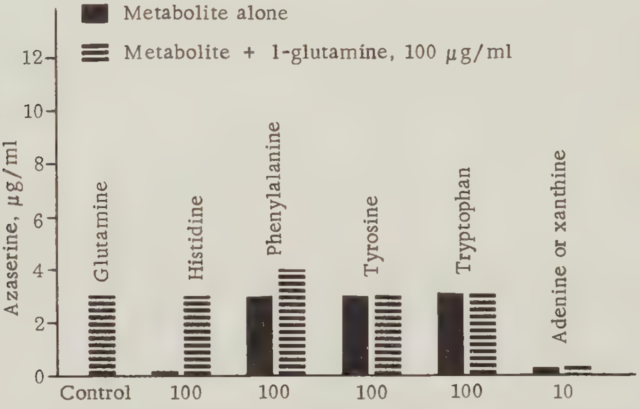


Fig. 8. The effect of certain metabolites on the inhibition of Escherichia coli by azaserine in glutamine-supplemented medium.

tyrosine, phenylalanine, and tryptophan and the degree of reversal was small. Neither histidine nor purines reversed azaserine under these condition (Fig. 8). Essentially the same results were obtained when the basal medium was supplemented with glutamic acid, isoleucine, threonine, methionine, arginine, or α-ketoglutaric acid. In occasional experiments isoleucine in variously supplemented media showed some degree of activity.

When the basal medium was supplemented with 1-tryptophan, either histidine or norvaline increased the concentration of azaserine required for complete inhibition thirty fold while a ten fold increase was required in the presence of leucine, isoleucine, valine, α-aminobutyric acid, aspartic acid, or arginine, each tested at 100 μg/ml (Fig. 9).

Histidine, isoleucine, norvaline, lysine, and citrulline gave an approximately thirtyfold reversal factor when the basal medium was supplemented with

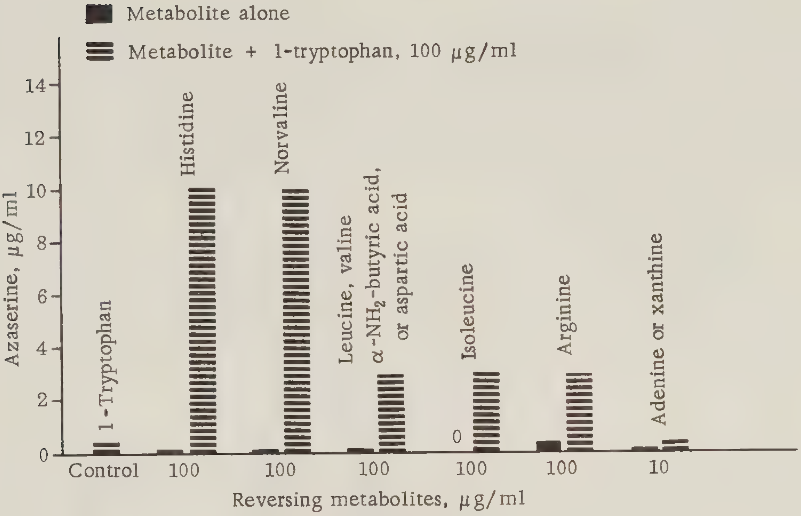


Fig. 9. The effect of certain metabolites on the inhibition of *Escherichia coli* by azaserine in tryptophan-supplemented medium.

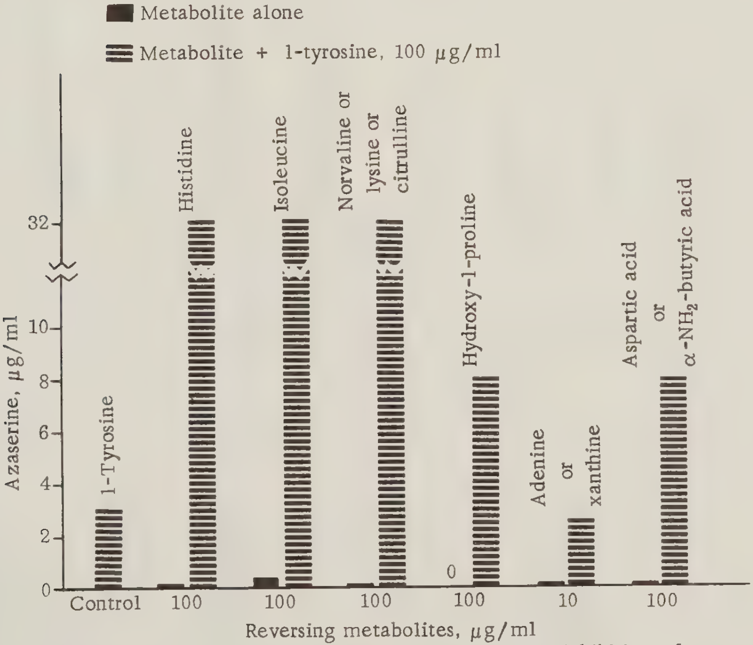


Fig. 10. The effect of certain metabolites on the inhibition of *Escherichia coli* by azaserine in the presence of tyrosine.

tyrosine. Hydroxy-1-proline showed less but significant azaserine-reversing activity (Fig. 10). Aspartic acid and α-aminobutyric acid which had only slight azaserine-reversing activity proved to be significantly more active in the presence of tyrosine, permitting growth in the presence of 8.0 μg azaserine/ml. Hydroxy-1-proline which alone has no azaserine-reversing activity enhanced the tyrosine reversal of azaserine, allowing full growth at 8.0 μg azaserine/ml.

The effect of metabolites on azaserine inhibition in the presence of both phenylalanine and histidine was determined (Fig. 11). In medium supplemented with 10 µg of each of these amino acids/ml, full growth occurred at 4 µg azaserine/ml but complete inhibition was obtained at 8 µg azaserine/ml. The addition of isoleucine, norvaline, methionine, tyrosine, or additional phenylalanine to this system permitted full growth of E. coli in the presence of 32.0 µg azaserine/ml. The addition of norleucine, α-aminobutyric acid, glutamine, homocysteine, tryptophan, or hypoxanthine allowed growth equal to control growth in the presence of 16 µg azaserine/ml.

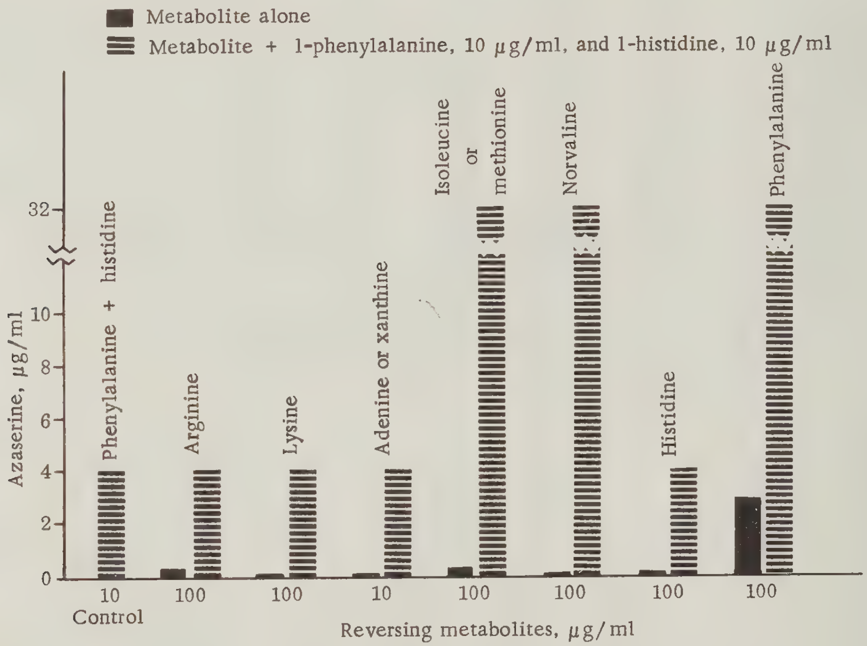


Fig. 11. The effect of certain metabolites on the inhibition of Escherichia coli in medium supplemented with both phenylalanine and histidine.

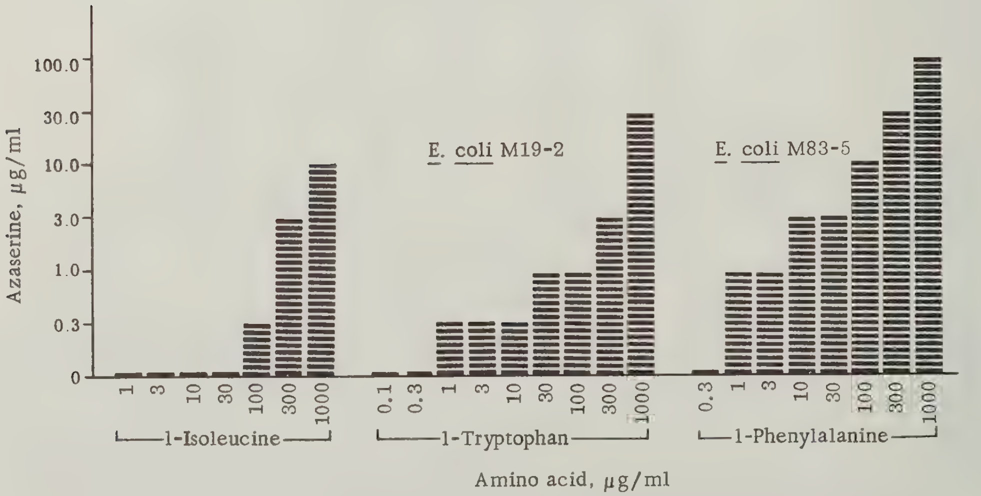


Fig. 12. The effect of azaserine on the growth of amino acid-requiring strains of E. coli.

Studies with mutant strains of E. coli. The effect of azaserine on the growth of three mutants of E. coli that require an exogenous supply of phenylalanine (strain M83-5), isoleucine (strain M9F-21), or tryptophan (strain M19-2) was determined. In all three strains (Fig. 12) it was found that in the presence of a concentration of amino acid just adequate for maximum growth, relatively small concentrations of azaserine completely suppressed growth. In the presence of large concentrations of the required amino acid larger concentrations of azaserine were required for complete inhibition. These data support the opinion of Kaplan et al. [14] that azaserine does not inhibit the biosynthesis of amino acids but interferes with their utilization. Furthermore, it was found that when these three mutant strains are growing on medium supplemented with just enough of the essential amino acid to support maximum growth, inhibition by azaserine is prevented by the same metabolites as in the case of the wild type E. coli.

DISCUSSION

The reversal of azaserine action by amino acids appears to be a generalized microbial event. In actively growing cultures of both Kloeckera brevis [11] and E. coli [9, 11, 12, 22] azaserine inhibition is markedly antagonized by amino acids. Our observations substantiate these findings and extend the phenomenon to include the Gram-positive bacterium, S. faecalis. Similarly, the reversal of threshold azaserine inhibition by purines reported by Bennet et al. [9] appears to be verified on the basis of our studies with E. coli, S. cervisiae, and S. faecalis. Failure of other investigators [14, 23] to confirm this observation may be due to the facts that individual purines alone reverse only very small concentrations of azaserine and variations in test procedures may have precluded successful demonstration of reversal.

It has been suggested by Kaplan et al. [14] and confirmed here that azaserine does not inhibit amino acid synthesis in E. coli. Since Tomisek [8] has clearly proven that this antibiotic does inhibit purine synthesis in E. coli, it must be concluded that amino acids reverse azaserine inhibition of E. coli by alleviating in some way the blockade of de novo purine synthesis. Furthermore, Tomisek et al. [24] have demonstrated that azaserine inhibition of de novo purine synthesis (as indicated by inhibition of 5'-phosphoribosyl-5-amino-4-imidazolecarboxamide accumulation in the metabolically deficient E. coli B96 [25]) can be reversed by phenylalanine and tryptophan not only in whole, actively growing cells but also in ultrasonically prepared cell-free material. This observation, incidentally, demolishes the proposal that aromatic amino acids antagonize azaserine action in some nonspecific way such as interference with either the penetration of the antibiotic through the cell membrane or the binding of azaserine by a cell-wall component [14].

If inhibition of de novo purine synthesis is the prime mechanism of action of azaserine in E. coli, it would seem likely that purines, nucleosides, or nucleotides (the ultimate products of the blocked reaction) would be efficient reversing agents. Since in E. coli they demonstrate only modest azaserine-reversing activity at best, we must consider alternate possibilities. The possibility that azaserine does not inhibit de novo purine synthesis is extremely unlikely in view of the wealth of data to the contrary [8-10,23,24,26,27] and the fact that purines are potent azaserine-reversing agents in S. cerevisiae. The possibility that purines are unable to pass through the cell membrane in azaserine-inhibited cells is unlikely since purines do reverse azaserine inhibition of growth. The possibility that some of the intermediates in the nucleotide synthetic pathway between formylglycinamidine ribotide and inosinic acid are essential to the formation of other products which in turn would be necessary in combination with nucleotides for total reversal is unlikely in absence of proof that such nonpurine products do exist. Lastly, the possibility that inhibition of de novo purine synthesis by azaserine is a consequence of azaserine inhibiting somewhere else and this inhibition being reflected as inhibition of de novo purine synthesis is conceivable.

Evidence exists that azaserine inhibits de novo purine synthesis as a consequence of its functioning as an antimetabolite for glutamine [26,28,29]. The considerable azaserine-reversing activity of glutamine in bacteria is consistent with this concept although one could expect a broader range of reversal. Failure to obtain truly competitive ratios over broad concentrations may be a consequence of irrevisible binding between azaserine and enzyme as suggested by Buchanan [27].

Nonetheless, combinations of other amino acids, notably phenylalanine and histidine, in *E. coli* do reverse extremely large concentrations of azaserine and appear to do so competitively. If azaserine is functioning as a glutamine antagonist, reversal by amino acid combinations (excluding glutamine) can be interpreted as a result of increased synthesis of the enzyme(s) required for transfer of glutamine γ nitrogen as a consequence of direct amino acid participation in the formation of the enzyme, increased synthesis of the same enzyme(s) as an indirect consequence of nonspecific amino acid action, or stimulated biosynthesis of unidentified material(s) (possibly a cofactor?) essential to the enzymatic transfer of the nitrogen atom from the glutamine.

ACKNOWLEDGMENTS

The interest and advice of Drs. R. Wallace Brockman, Frank M. Schabel, Jr., and Howard E. Skipper is gratefully acknowledged. The invaluable technical assistance of Mrs. Barbara Quinnelly, Miss Marilyn Kangelos, Mr. William Logothetis, Mr. Harold Pierce, Mrs. Ann Seawel, and Mrs. Dorothy Harper is appreciated.

SUMMARY

The reversal of azaserine inhibition by amino acids, notably tyrosine and phenylalanine, appears to be a generalized microbial phenomenon. In *Escherichia coli* the reversing activity of phenylalanine is greatly enhanced by histidine, xanthine, adenine, arginine, ornithine, and norvaline but not by glutamine. The azaserine-reversing activity of tyrosine is enhanced by histidine, isoleucine, norvaline, lysine, and citrulline but not by either glutamine or purines. In the presence of small concentrations of phenylalanine and histidine, large concentrations of azaserine were reversed by isoleucine and methionine.

Data are presented which substantiate the conclusion that azaserine does not inhibit the biosynthesis of amino acids.

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DISCUSSION

DR. HAHN: At the beginning of your lecture you mentioned very briefly certain reasons why work on this problem in liquid culture might lead to misleading results. Would you care to comment on this please?

DR. PITTILLO: In a recent publication Kaplan, Stock, and Riley reported some beautiful and elaborate work wherein they showed that as a consequence of filament formation in *E. coli* resulting from exposure to azaserine, the turbidity measurments were not a true indication of the number of viable cells in the tube, the viable cells being measured by standard plating-out procedures.

BIO-ASSAY AND SPECTROPHOTOMETRIC STUDIES ON THE INTERACTION OF KANAMYCIN AND NEOMYCIN WITH NUCLEIC ACIDS

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and Alfred H. Lawton

Veterans Administration Center, Bay Pines, Florida

INTRODUCTION

Davis, MacIntyre, and Lawton [1] have reported that neomycin and kanamycin have the capacity either to desorb or prevent the initial uptake of large quantities of vitamin B_{12} (tagged with Co^{60} for easy identification) previously adsorbed to or in contact with the resting cells of certain lactobacilli. Highly polymerized preparations of nucleic acids, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) were also demonstrated to possess this property [2]. The degree of polymerization and amount of nucleic acid influence the extent of microbial desorption or inhibition capacity. In view of the significance of the nucleic acid content of the bacterial cell and cell surface and their mutual effect on the microbial disposition of vitamin B_{12}, experiments were undertaken to study any interaction of neomycin and kanamycin with highly polymerized preparations of DNA and RNA. The results of such studies are described in this paper.

MATERIALS AND METHODS

Vitamin B_{12} Co^{60}. The vitamin B_{12} containing Co^{60} employed in this study had a specific activity of approximately 1000 μC/mg. The microbiological potency was measured previously by the method of Skeggs [3]. The Co^{60} vitamin B_{12} used in this study was supplied through the courtesy of Dr. Charles Rosenblum of Merck and Co., Rahway, N.J.

Cultures of *Lactobacillus leichmannii* ATCC 4797. These were supplied through the courtesy of Dr. David Hendlin of Merck and Co.

Resting cells. The preparation of resting cells was described previously by Davis, Duvall, and Chow [4]. The resting cells were kept at 4–8 C for approximately 5–7 days with minimum exposure to room temperature.

Kanamycin sulfate.* This antibiotic was prepared by dilution with water or 0.85% sodium chloride solution as required to yield a solution containing 100 mg/ml and served as stock solution. Further dilutions were prepared as required.

Neomycin sulfate.† See discussion of kanamycin sulfate for preparation.

Spectrophotometry. Spectrophotometric readings were performed with a Beckman DK-2 recording spectrophotometer.

Bio-assay technique. Varying amounts of antibiotics were added to amounts of nucleic acids previously demonstrated to prevent the microbial adsorption of 0–90% of the radiovitamin. The supernatant fluids of this interacting system were then mixed with 10 mμg of Co^{60} vitamin B_{12}. Resting cells, 10^9 in 0.85% saline solution, were added. The suspension was shaken thoroughly, then centrifuged.

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*The trade name of Bristol Laboratories, Inc., for kanamycin sulfate is Kantrex.

†The trade name of the Upjohn Co. for neomycin sulfate is Mycifradin Sulfate.

The harvested resting cells were washed with distilled water after thorough draining. The radioactivity adsorbed to the resting cells due to the vitamin B_{12} containing Co^{60} was measured with a thallium-activated, sodium iodide well, scintillation counting system.

RESULTS AND DISCUSSION

Figure 1 presents the curves derived from plotting the adsorption or uptake of Co^{60} vitamin B_{12} by a constant number of resting cells (10^9) of L. leichmannii against the amount of kanamycin (μg/tube) added. The data demonstrate that increasing amounts of antibiotic added to a constant amount of nucleic acid result in decreased inhibition of microbial adsorption of Co^{60} B_{12} by the nucleic acids. If kanamycin is not incorporated into the system, approximately 90% inhibition of bacterial uptake results when 200 μg of RNA are added to the resting cells and Co^{60} B_{12}. The addition of approximately 500 μg of kanamycin neutralizes the RNA inhibition of Co^{60} vitamin B_{12} uptake by interaction with the nucleic acid. When 1 mg of DNA is added approximately 80% inhibition of microbial uptake of Co^{60} B_{12} results. This inhibition can be reversed by 500 μg kanamycin. Further addition of either kanamycin or neomycin results first in complete interactions with the nucleic acids and second in excess of antibiotics with their resultant inhibitory effect of microbial uptake. The above curves are the average of three determinations performed on 3 different days. The unexpected and unexplained finding in the DNA curves at the 400 μg level is reproducible. In this curve increased microbial uptake results with increased interactions up to approximately 300 μg but is depressed several hundred counts at 400 μg. After this level the curve resumes its expected course.

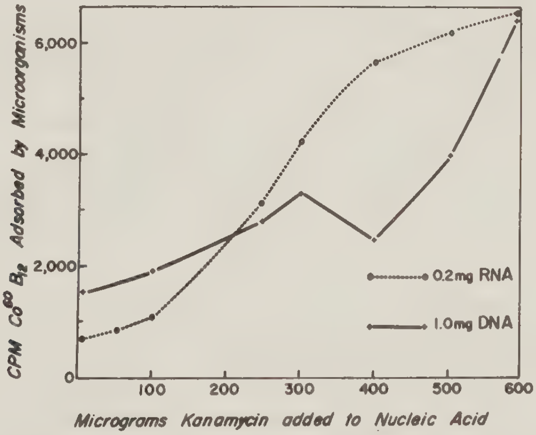

Fig. 1. Effect of interaction of kanamycin with nucleic acids on the microbial adsorption of Co^{60} vitamin B_{12}.

To determine whether neomycin reacts in a similar manner to kanamycin, plots of the uptake of a constant amount of Co^{60} vitamin B_{12} against increasing amounts of neomycin (0–50 μg) added to one mg of highly polymerized DNA were made. The results are plotted in Fig. 2. The curve clearly indicates that increased amounts of antibiotic added to a constant amount of nucleic acid result in increased microbial uptake of the radiovitamin. The interaction of the nucleic acid and antibiotic neutralizes their mutual inhibitory capacity of microbial uptake of Co^{60} vitamin B_{12}. Figure 3 presents the results of experiments similar

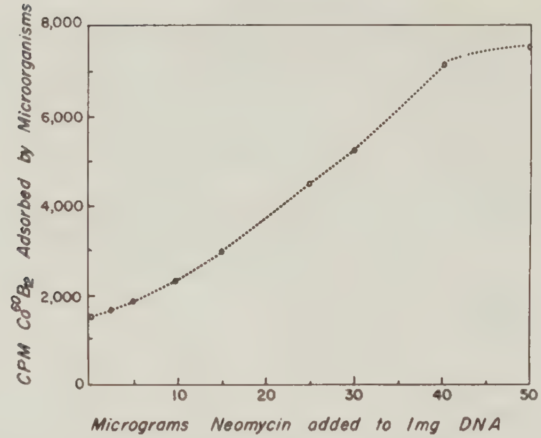

Fig. 2. Neutralization of inhibition of microbial adsorption of Co^{60} vitamin B_{12} by interaction of neomycin with DNA.

to that described in Fig. 2, except that RNA was employed in place of DNA. The resultant curve indicates that approximately 8 μg of neomycin added to 200 μg of RNA will completely neutralize the inhibition of microbial adsorption of vitamin B_{12} possessed by the RNA. This is evident even at the 3-μg level.

To determine whether the precipitation and interaction of DNA by kanamycin and neomycin were effected or influenced by pH, preparations of DNA (0.5 mg/ml in saline) were treated with varying amounts of 0.02–0.2 N sulfuric acid solutions. Observations were made to determine the pH at which the first appearance of precipitation of DNA occurred. No appreciable cloudiness or turbidity occurred until approximately pH 1.8. Marked precipitation occurred at pH 1.55. Since kanamycin and DNA are slightly acidic either singly or in combination their precipitation was not due to pH.

Dialysis experiments indicated that neomycin or kanamycin will diffuse rapidly through a cellophane membrane and completely precipitate the DNA within the membrane. This combination of the nucleic acid and antibiotic occurred rapidly. When 2 mg of DNA are placed inside the membrane and 1 mg of neomycin or kanamycin placed outside, the DNA is almost completely reacted

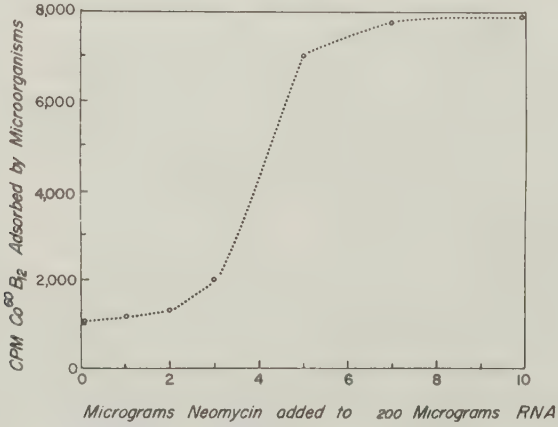

Fig. 3. Neutralization of inhibition of microbial adsorption of Co^{60} vitamin B_{12} by interaction of neomycin with RNA.

with the antibiotic after 3 hr of shaking. The ratio of nucleic acid to antibiotic resulting in most rapid and quantitative reaction is approximately 5 : 1. As little as 20 μg neomycin will result in demonstrable precipitation of 2 mg of DNA or RNA.

Spectrophotometric measurements were made employing solutions of DNA, kanamycin, and a mixture of the two in saline. Figure 4 illustrates typical curves resulting from DK-2 recordings of the three solutions. The highly polymerized DNA had a maximum absorption at approximately 260 mμ (Fig. 4). Large amounts of kanamycin equivalent to 2.5 mg/ml also had a maximum absorption at 260 mμ. Since the kanamycin was in great excess, Fig. 4 represents the optical density of the large amount unreacted kanamycin and a smaller amount of DNA.

There is no doubt that other antibiotics not studied would behave similarly to kanamycin and neomycin. It well may be that the interaction of selected antibiotics and nucleic acids at the cell-surface level play some role in the mechanism of antibiotic action. Further study is necessary to determine whether this combination of antibiotics and nucleic acids is of significance in the action of certain antimicrobial agents.

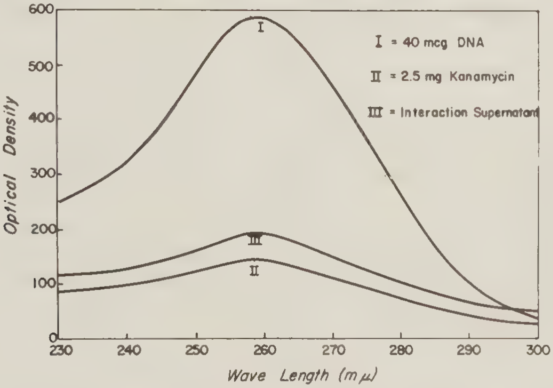

Fig. 4. Ultraviolet absorption spectra of DNA, kanamycin, and the supernatant of a mixture of their interaction.

SUMMARY

Kanamycin and neomycin will interact with ribonucleic and deoxyribonucleic acids. Interaction of neomycin with ribonucleic acid can be detected at approximately the 1-μg level. Kanamycin will show significant interaction with the nucleic acid at approximately the 50-μg level. Spectrophotometric studies indicated marked interaction of nucleic acids with kanamycin.

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ANTIBIOTIC INHIBITORS. THE EFFECT OF MULTIVALENT METALLIC CATIONS ON INTESTINAL ABSORPTION AND IN VITRO ANTIBACTERIAL ACTIVITY OF OXYTETRACYCLINE

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INTRODUCTION

Certain multivalent cations are known to exert an inhibitory effect in vitro on the antibacterial activity of the tetracycline antibiotics. These metals include iron (ferrous and ferric), magnesium, manganese, aluminum, and calcium [1-4]. The suppressive action of calcium and magnesium on the antibacterial efficacy of oxytetracycline‡ (OTC) has been confirmed in this laboratory [2].

Rat and human studies have shown that tetracycline blood concentrations are lower when dicalcium phosphate is ingested with the antibiotic [5,6]. Studies by the present authors have shown that Ca^{++} administered concurrently with OTC into the ligated duodenal loop of chicks depresses absorption of antibiotics, as evidenced by lowered serum, urine, and liver antibiotic concentrations and by greater retention of OTC in the loop as compared with controls receiving antibiotic alone [7]. This phenomenon can be applied to enhance the effectiveness of orally administered antibiotics in the treatment of certain poultry diseases. Use of rations in which dietary calcium has been temporarily reduced during the administration of tetracycline antibiotics has permitted greatly increased antibiotic serum concentrations [8,9] and has augmented the effectiveness of antibiotics against disease [10].

There are indications that one of the trivalent ions, Al^{+++}, may influence intestinal absorption of chlortetracycline§ in humans [11,12].

It was of interest to determine the degree of parallelism between the effect of ions on suppression of in vitro antibacterial activity of antibiotics and their effect upon retardation of intestinal absorption. It also was of interest to determine whether di- and trivalent ions might differ in these capacities. Such a study, which could throw additional light on the mode of action of cation inhibition of antibiotics, is not without practical significance inasmuch as manganese, magnesium, and iron are normal dietary components.

MATERIALS AND METHODS

In vitro studies. The antibiotic-cylinder plate-diffusion assay method was used to determine the influence of a range of concentrations of Ca^{++}, Mg^{++}, Mn^{++}, Fe^{+++}, and Al^{+++} upon antibacterial activity of oxytetracycline against Pseudomonas aeruginosa (1014), Bacillus cereus, var. mycoides (1245), Escherichia coli (1001), and Micrococcus pyogenes var. aureus (1052). The following procedure was used to test each concentration of each cation. Four cylinders were spaced equidistant on a seeded agar plate. The cationic solution was placed in one cylinder; into a second was placed the antibiotic in a concentration known from preliminary tests to provide a maximum zone of inhibition; and the antibiotic plus the cation were placed in the third and fourth cylinders. Allowing for 1 : 1 dilution of the cation solution by the solution of antibiotic, the range of concentration of cations was from 0.00004 to 0.8 M. The plates were incubated at 37 C for 18 hr and the width of zones of inhibition measured with a Bausch & Lomb Balopticon zone projector.

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†Present address, Bristol Laboratories, Syracuse, New York.

‡Terramycin, Chas. Pfizer & Co., Inc.

§Aureomycin, American Cyanamid Company.

In vivo studies. Three experiments were conducted with an aggregate of 105 New Hampshire chickens averaging 950 g in weight. The duodenal-loop-ligation technique [7] was used to determine the effect of Ca^{++}, Mg^{++}, Mn^{++}, Fe^{+++}, and Al^{+++} on OTC absorption. The intestinal contents were expressed from the segment before applying the second literature. In all cases 4 ml of solution containing 1.5 mg OTC/ml was injected into the isolated segment. The cation concentration ranged from 0.0001 to 0.4 M.

Pretreatment blood samples were obtained 30 min prior to injection and posttreatment samples were collected 2 hr postinjection from the wing vein and 4 hr postinjection from the heart, after which the birds were sacrificed. Urine samples were collected at 2 and 4 hr postinjection directly into Petri dishes by insertion of a small speculum into the cloaca.

RESULTS

Figure 1 shows the molar concentrations of various multivalent cations in vitro required to yield a 50% reduction of the OTC activity against the four test species of bacteria. For the three divalent ions these values ranged from approximately 10^{-2} to 10^{-1} M. For the two trivalent ions the range was in the vicinity of 10^{-4} M.

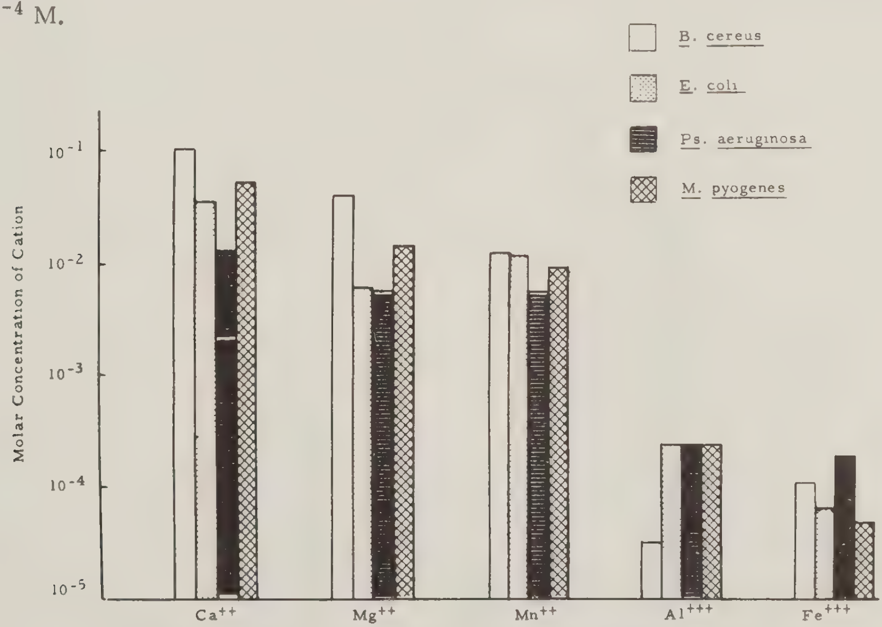

Fig. 1. The molar concentrations of various multivalent cations required to reduce oxytetracycline antibacterial activity by 50%.

Effect of these ions on intestinal absorption is illustrated in Figs. 2 and 3, which depict the serum and urine OTC concentrations as influenced by the cations simultaneously injected with antibiotic into the ligated duodenal loop. Initial concentrations of 0.01 M of Ca^{++}, Mg^{++}, and Mn^{++} in the segment reduced both serum and urine levels of OTC approximately 50%. The serum levels indicate that little or no inhibition of intestinal absorption occurred with divalent ions in concentrations of 0.001 M. Al^{+++} and Fe^{+++} were more inhibitory than the divalent ions as, at initial concentration of 0.001 M, they lowered serum levels of the antibiotic by 50% and lowered urine concentrations even more than this. The serum levels indicate that inhibition did not occur with either trivalent cation at 0.0001 M.

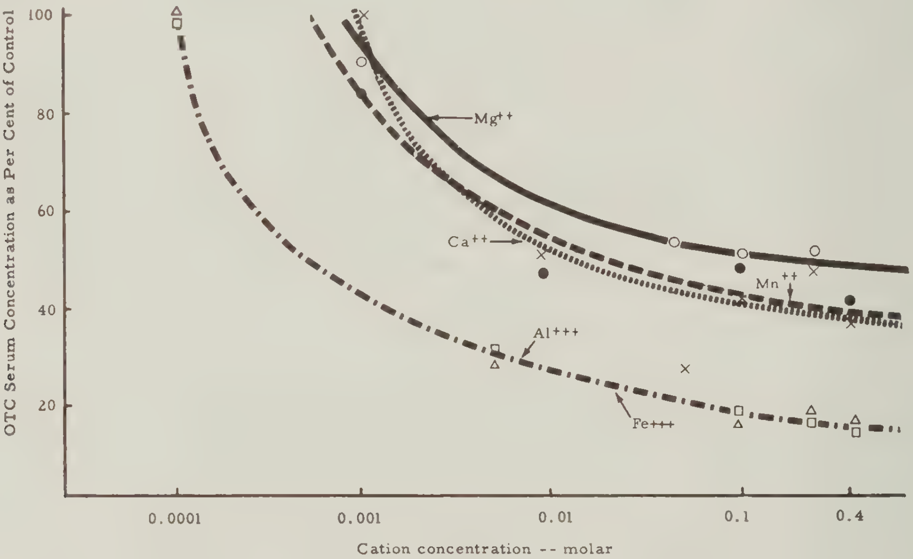

Fig. 2. Oxytetracycline serum concentration as influenced by cations following simultaneous injection with antibiotic into the ligated duodenal loop of chickens. (Average of 2- and 4-hr posttreatment specimens.)

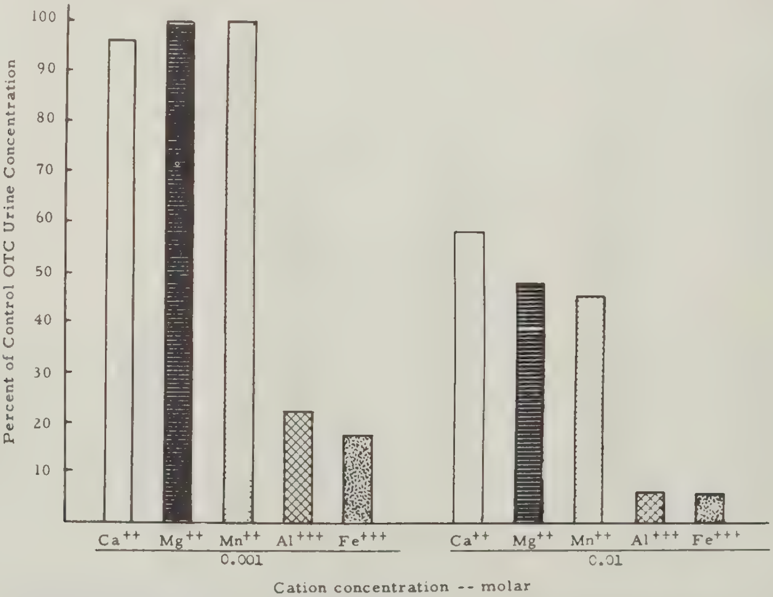

Fig. 3. Oxytetracycline urine concentrations as influenced by cations following simultaneous injection with antibiotic into the ligated duodenal loop of chickens. (Average of 2- and 4-hr posttreatment specimens.)

DISCUSSION AND CONCLUSIONS

The inhibition of antibacterial action of oxytetracycline by certain divalent and trivalent cations was confirmed. The trivalent ions, Fe^{+++} and Al^{+++}, were 100 to 1000 times more inhibitory than the divalent ions, as assessed by concentrations necessary to give a 50% reduction of in vitro activity against four species of organisms.

The in vitro inhibitory activity of these ions was closely paralleled by their ability to retard absorption of oxytetracycline through the wall of the isolated duodenal loop of the chicken. The divalent ions, Ca^{++}, Mg^{++}, and Mn^{++} were similar in activity as all three reduced serum and urine concentrations of OTC by 50% when the initial ion concentration present in the loop was 0.01 M. The trivalent ions were 10 times, or more, as effective as the divalent ones in retarding intestinal absorption.

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INTRODUCTION TO THE PANEL ON MODES OF ACTION OF ANTIBIOTICS

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It is a surprising fact that twenty years after the advent of widespread usage of antibiotics, the biochemical and physiological mechanisms whereby the antibiotics exert their cytotoxic effects remain, in general, illusory. Much of the difficulty lies in distinguishing between direct, immediate, and primary effects of the antibiotics and secondary damage to the cell resulting from the primary lesions. This objection presumably can be met in part, at least, by use of cell-free extracts. However, reports on inhibition of enzymatic reactions in bacterial cell-free systems are rare. Further, many publications have reported results indicating various inhibitory activities of the antibiotics, but close examination of the data presented reveals numerous technical discrepancies, chief among which is the use of concentrations of inhibitor far in excess of those necessary for inhibition of growth of the organisms. Such results, though of some significance, must be viewed cautiously.

Another factor often overlooked is rigid control of pH throughout the course of the experiment. It is a well-known fact that a change of even one pH unit, in many instances, may increase or decrease from ten- to a hundredfold the minimal inhibitory concentrations of antibiotics. Further, it would seem desirable, before inhibition of a given reaction by an antibiotic is presented as the primary mode of action of the compound, to investigate this inhibition in resistant cells derived from the parent sensitive strain. Obviously, the reaction of the resistant cell to the antibiotic should differ from that of the sensitive one. In addition, it is necessary that the inhibition clearly be of such vital significance to the total economy of the cell that it should not multiply or survive without the products of the inhibited reaction.

However, when these and other considerations are taken into account the disturbing situation still prevails. The modes of action of the various antibiotics remain relatively obscure. Obviously, it is far easier to state the problem than it is to solve the problem. Nevertheless, as a result of work published in recent years, and particularly by the members of this panel who are well aware of all pitfalls, it is now possible to present some working hypotheses which, though not pinpointing specific and definitive loci of the inhibited reactions, at the very least, indicate various avenues of bacterial physiology wherein the metabolic block might be expected to occur.

PERMEABILITY AND THE MODE OF ACTION OF ANTIBIOTICS

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The importance of permeability for the life of the cell is readily apparent. Living cells exist in constantly changing environments. They are able to survive and maintain their integrity in these environments because they have barriers which exclude the free passage of external solutes into the cell, and which prevent the passage of internal solutes out of the cell. These barriers are called membranes. The integrity and smooth functioning of these membranes is critical for the life of the cell. Because these membranes exist at the periphery of the cell, where they are constantly coming into contact with the outside world, they are constantly being bombarded with potentially dangerous stimuli to which they must respond in a reproducible and significant way.

Membranes are usually described as semipermeable, since, although they are freely permeable to water and many nonionic polar molecules, they are usually not freely permeable to inorganic and organic ions and many other polar and nonpolar molecules. However, these latter substances do get into cells, not by a mere passive diffusion, but by a process called active transport, since energy is usually expended. In addition to energy requirements, active transport mechanisms usually have high specificity, so that, for instance, potassium ions and sodium ions do not move into cells equally well. In recent years the word "permease" has been coined to help explain certain stereospecific active transport situations in microorganisms, although it seems to me that this word may conceal more than it reveals about the nature of these processes.

Membrane semipermeability enables cells to exclude many types of molecules under certain conditions and take them up under other conditions. This gives cells a useful metabolic control system and enables them to adjust readily to changing environments. Membrane semipermeability also makes it possible for cells to retain within them certain molecules, such as important precursors which are vital for their own activity, which would otherwise be lost because of diffusion outward. It seems to me that this latter aspect of permeability may be of great importance for the continuing existence of the cell.

Drugs which act on the bacterial cell membrane are of at least two types. There are the surface-active agents, such as the quaternary ammonium compounds, and the antibiotics tyrocidin and polymixin, which act directly on the cell membrane in nongrowing cells [1, 2]. When these substances are added to bacteria, there is a direct combination of the surface-active agent with the membrane, the membrane is disorganized, and the soluble cell contents leak out. These drugs are usually directly bactericidal to nongrowing cells. It is not clear whether the killing is due to the mere loss of the soluble cell-pool materials or whether it is due to a disorganization of the cell membrane, making it nonfunctional for active transport and other permeation processes. However, the situation is relatively simple.

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*Research of the author is supported by grant E-3722 from the National Institutes of Health, U.S.P.H.S.

Recently there have been several reports of antibiotics which had no effects on permeability in nongrowing cells but did affect permeability in growing cells (streptomycin: Anand and Davis, [3]; novobiocin: Brock and Brock, [4]). These antibiotics were not bactericidal to nongrowing cells, but did kill growing cells.

Our own work in this area began when we were studying the effects of novobiocin and erythromycin on beta-galactosidase synthesis in an inducible strain of *Escherichia coli*. Although results with erythromycin were straightforward, we found with novobiocin that beta-galactosidase assays on nontoluenized cells after antibiotic treatment were higher than expected. Because it was known [5], that permeability of the cell to the beta-galactosidase substrate varied widely with growth conditions, we postulated that our results might be due to an effect of novobiocin on the permeability of the cell. This was also suggested by the observation that novobiocin-treated Gram-negative cells became unstainable by methylene blue, indicating a loss of basophilic substances. Also, Gram-positive cells lose their Gram-positivity when treated with novobiocin, and Gram-positivity is considered to be due to the impermeability of these cells to the decolorizing agent.

To analyze further these permeability relationships with novobiocin, we used strain ML 35 of *E. coli*. This strain forms beta-galactosidase constitutively at very high levels. However, the assays of whole cells of this strain with o-nitrophenyl-beta-D-galactoside, the usual chromogenic beta-galactosidase substrate, are quite low. Monod has postulated that there is a specific permeation enzyme, a permease, which functions in the uptake of beta-galactosides. Strain ML 35 is then considered to lack this enzyme, and therefore the chromogenic substrate is excluded from the cell, and assays of whole cells reveal negligible beta-galactosidase activity. However, if the cells are treated with toluene, the permeability barrier is broken, and extremely high beta-galactosidase activities are obtained.

It was a simple matter to add antibiotics to growing cells of *E. coli* ML 35 and measure beta-galactosidase activity, at various intervals, in whole cells. This gave us a measure of the nonspecific effect on permeability of each antibiotic. We therefore screened a number of antibiotics for changes in activity (Table 1). Streptomycin and novobiocin seem to stand apart from the others, since they bring about marked increases in permeability, without accompanying lysis.

Detailed studies on the action of novobiocin on growth, viability, and beta-galactosidase activity were undertaken [4]. It was found that after the antibiotic was added, growth (as measured by optical density or protein content) continued to increase, while viability began to drop without a lag, and beta-galactosidase activity of whole cells rose. The fact that the increase in beta-galactosidase activity and decrease in viable count were parallel led us to postulate that a permeability change was responsible for the loss in viability. These changes only occurred in growing cells. These facts, together with a number of other miscellaneous observations, prompted us to postulate that novobiocin might be acting by inhibiting the synthesis of the cell membrane. Anand and Davis [3] have made a similar postulate for streptomycin.

Although this technique is useful for screening antibiotics for permeability effects, it has certain pitfalls. The beta-galactosidase activity of ruptured cells of this strain is very high, about 170 times the activity of whole cells. Even a small amount of cell lysis would result in a marked increase in activity, even though this activity would be a small fraction of the total activity possible. Optical

Table 1. Change in Beta-Galactosidase Activity of Whole Cells (Nontoluenized) During One Hour Growth Inhibition

Agent added	Growth Inhibition, %	Increase in beta-galactosidase specific activity, %
Control	0	0
Chloramphenicol, 50 μg/ml	95	24
Erythromycin, 1000 μg/ml	95	28
KCN, 10^{-3} M	95	13
NaN_3, 10^{-3} M	67	17
Polymyxin B, 200 μg/ml	lysis	1250
Penicillin G, 600 μg/ml	lysis	7000
Streptomycin, 50 μg/ml	85	360
Novobiocin, 1000 μg/ml	88	345

E. coli ML 35 growing in a salts-glycerol medium. Antibiotics added at OD 0.1115 (420 mμ), assayed for beta-galactosidase at 0 and 1 hr. Beta-galactosidase measured by hydrolysis of ortho-nitrophenyl-beta-D-galoctoside (Brock and Brock, 1959) and specific activity calculated by dividing color OD by turbidity OD. Where lysis occurred, 0 time turbidity was used to calculate specific activity.

density readings might not reveal this lysis. With novobiocin we used several other criteria to show that this was a real permeability increase. The novobiocin-induced increase in enzymatic activity remained in the sediment when the culture was centrifuged at relatively low speed, whereas when lysis was induced by penicillin or sonication the bulk of the activity remained in the supernatant. Also, the sedimented cells could be resuspended in fresh medium and still reveal the increased activity. Further, a marked leakage of intracellular constituents could also be detected. Even with these additional facts, it is still necessary to consider the possibility that the whole phenomenon can be explained in terms of cell lysis, especially since Strominger and Threnn [6] have presented evidence that novobiocin induces the accumulation of cell wall precursors in Staphylococcus aureus.

Another example of the complications that can arise in using this technique for measuring effects of agents on permeability is shown by results we have obtained with azaserine [7]. When this antibiotic is added to growing cells of E. coli ML 35, after a delay of 1 hr, there is a sharp rise in beta-galactosidase activity in nontoluenized cells almost up to the level of toluenized cells. Irradiation of the cells with UV light produces a similar effect. This rise in activity is accompanied by a moderate lysis. Because both UV light and azaserine are known to induce the productive response in lysogenic cultures, it occurred to us that E. coli ML 35 might be lysogenic, and the marked rise in beta-galactosidase activity and moderate lysis might be due to bacterial virus production. We tested a large number of strains of E. coli and other enteric bacteria as possible hosts for an ML 35 virus, but were unsuccessful. It is possible that this strain might be a defective lysogenic organism which produces a nonvirulent virus. If this strain is lysogenic, it further complicates its use in antibiotic-induced permeability studies. However, there is no reason why the principle used here, the measurement of enzyme activity in a constitutive cryptic mutant before and after antibiotic treatment, could not be applied with other organisms and other enzymes.

From our data with E. coli, we had postulated that novobiocin inhibited the synthesis of the cell membrane. Data on the chemical anatomy of E. coli revealed, however, that a clear-cut morphological or chemical distinction between the cell wall and the cell membrane was not possible [8]. This made it difficult to devise experiments that would indicate unequivocally whether or not novobiocin inhibited cell membrane synthesis. In discussing this problem with Dr. Fred Hahn last spring, he made the suggestion that the best test organism for this study was Micrococcus lysodeikticus. A review of the literature showed this to be true. M. lysodeikticus has a very simple cell wall structure [9, 10]. The wall has no lipid, and the lack of phosphorus indicates absence of teichoic acid. It is high in hexosamine and glucose and has a limited complement of amino acids. It has no enzymes and no pigment. Further, the wall is completely digested by lysozyme. This makes possible the direct isolation of the cell membrane. The chemical composition of the membrane, as shown by Gilby et al. [11] is also quite distinctive and differs quite markedly from the composition of the wall. All of the carotenoid pigment of the cell is in the membrane. In addition there is present a large amount of lipid and protein. Present also is 26% carbohydrate, most of which is mannose; glucose and hexosamine being absent. A number of enzymes involved in oxidative metabolism are also in the membrane [12].

The high mannose content of the membrane was perhaps surprising in a structure that was usually considered to be lipoprotein. But it suggests a possible site of attack for an antibiotic such as novobiocin, which has an unnatural sugar in its structure. Gilby et al. [11] had already shown that C^{14}-glucose could be incorporated into the mannose of the membrane. Therefore, experiments were set up to see if novobiocin had an effect on this incorporation process, as a possible direct indication of its inhibition of membrane synthesis.

Unfortunately, the results obtained do not allow such a simple interpretation. In the experiments done, glucose-U-C^{14} was added to cells growing in a medium containing peptone, sodium acetate, and ribose. Novobiocin was always added

Table 2. Effect of Novobiocin on the Incorporation of Glucose-U-C^{14} into Various Cell Fractions

Component	Inhibition, %		
	0.1 μg/ml	1 μg/ml	10 μg/ml
Cold TCA insoluble (total)	30	63	66
Nucleic acid	30	61	–
Protein	31	49	–
Lipid	31	59	–
Cell wall	27	68	65
Cell membrane	37	41	61

Micrococcus lysodeikticus growing logarithmically at 32C in peptone — acetate — ribose medium of Gilby et al. (11), OD 0.400 (650 mμ) when antibiotic added. Five minutes after antibiotic, 0.1 μC/ml of glucose-U-C^{14} added (specific activity, 1 μC/mmole), 1-ml samples taken after 1 hr, processed by method of Roodyn and Mandel (13) for RNA protein, and lipid incorporation. Cell walls isolated by procedure of Park and Hancock (17) or Brock (unpublished). Membranes isolated by procedure modified from Gilby et al. (11). All samples filtered on Millipore type HA membrane filters, counted on Nuclear Instruments scaler with Geiger tube.

5–10 min before the glucose. After 1 hr, samples were taken to determine the distribution of radioactivity in various fractions, using the membrane filter technique of Roodyn and Mandel [13]. Cell membranes and cell walls were prepared, filtered, and counted. The preliminary results (Table 2) show that novobiocin inhibited incorporation equally into all fractions. Thus, it appears that novobiocin inhibits some early step in glucose assimilation, possibly uptake or activation, rather than the specific glucose incorporation into the cell membrane. Similar effects have also been reported recently by Hancock [14] for streptomycin. Since the enzymes involved in oxidative reactions are in the cell membrane [12], it is possible that glucose assimilation is being inhibited indirectly as a result of membrane disorganization. It seems clear, at any rate, that the simple hypothesis that novobiocin specifically inhibits membrane synthesis cannot be verified using glucose incorporation, since glucose incorporation, in general, is inhibited.

To analyze further the novobiocin inhibition of glucose incorporation, the uptake of C^{14}-glucose into the cold TCA-insoluble material and into the cold TCA-extractable pool was measured in growing cells. There is no inhibition of uptake into the pool (Fig. 1). However, 30 min after antibiotic addition there is no further incorporation of glucose into the TCA-insoluble fraction. These

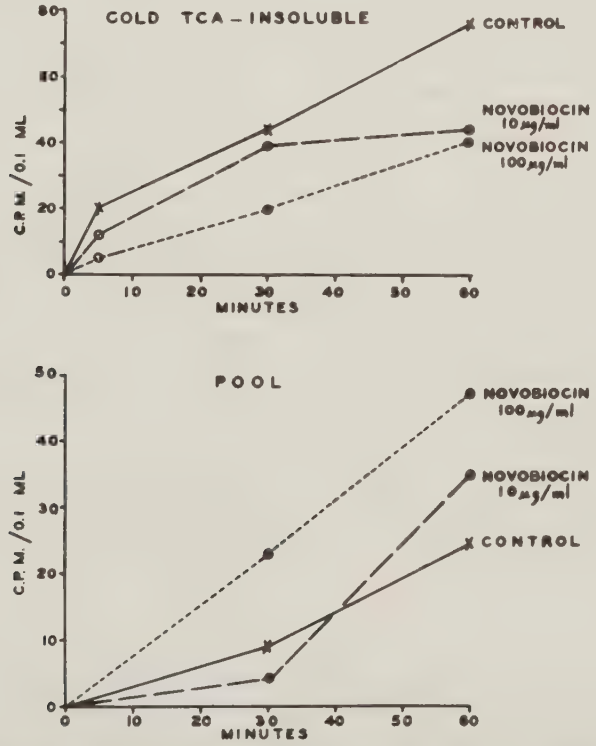

Fig. 1. Effect of novobiocin on incorporation of glucose-C^{14} into cold TCA-soluble (pool) and cold TCA-insoluble fractions in growing cells of Micrococcus lysodeikticus.

See Table 2 for conditions of growth. Five minutes after antibiotic, 0.1 μC/ml of glucose-U-C^{14} added (specific activity 10 μC/mmole), 0.1-ml samples taken at intervals and diluted into 1 ml cold phosphate-saline and filtered immediately, then washed twice with phosphate-saline. Parallel 0.1-ml samples diluted into 1 ml cold 5% trichloroacetic acid, kept in cold 1 hr, then filtered and washed with cold 5% trichloroacetic acid. Counted as in Table 2.

results seem to show that novobiocin is disrupting some process which only secondarily leads to inhibition of glucose incorporation. If the same experiment is done with nongrowing cells in phosphate buffer, there is little inhibition by novobiocin of either uptake into the pool or incorporation into cold TCA-insoluble material. Obviously many more experiments remain to be done, and I present these preliminary results here to acquaint anyone else wishing to work on membrane synthesis with the possible complications involved.

So far as I know, the structure of novobiocin is unique, and there are no other related antibiotics or natural products. The molecule has two acidic functions, the phenolic group and the enolic group, a peptide bond, a derived coumarin, and an unusual sugar. The sugar is especially interesting, since there is an o-carbamyl group on the 3 position. If this carbamyl group is moved to the 2 position, the inactive compound, isonovobiocin, is formed. I feel that this marked stereospecificity of the active molecule indicates a highly specific structural or enzymatic site of action. Although certain similarities of action of streptomycin and novobiocin have been mentioned here, these two antibiotics are quite different chemically, even though both have unnatural sugars.

Another example of the effect of an antimicrobial agent on permeability is found in the work of Schaefer [15]. He showed that isoniazid brought about changes in growing cells of Mycobacterium tuberculosis which resulted in the loss of acid-fastness and an increased ability to reduce a tetrazolium dye. These changes were accompanied by a loss in viability of the cells. If nongrowing cells were used, none of these changes were noted. These results are quite analogous to our results with novobiocin. Schaefer has also explained his observations as due to the alteration of a permeability barrier.

In conclusion, we might speculate briefly on the mechanism of synthesis of cell wall and cell membrane. When the cell grows, the existing cell wall must be stretched in some way and new material added in the interstices. If the synthesis of new material is inhibited and stretching continued, gaps in the cell wall will soon appear, and the cell contents can be forced out through these holes. This is apparently what happens during penicillin lysis [16]. In the same way, the cell membrane must stretch with new material being added. If the synthesis of this new material were inhibited, an increase in permeability of the cell would result, without frank lysis, since the wall remains intact, although the intracellular pool could leak out.

We have no direct evidence for such inhibition of membrane synthesis, but our work has just begun, and there are a wide variety of possible experiments which can be done. It would seem quite reasonable to assume, on the basis of present knowledge, that some bactericidal antibiotics might act by inhibiting membrane synthesis.

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STUDIES ON MODE OF ACTION OF STREPTOMYCIN*

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By definition, a medically important antibiotic to be used other than topically is one which will kill or inhibit the growth of pathogenic microorganisms but which will not affect the host cells.

A large body of documentation exists pointing out the essential unity of biochemical pathways in forms of life as diverse as microorganisms and humans, As a preliminary guess, it would therefore appear unlikely that organism-specific antibiotics could exist at all. Fortunately, the unlikelihood of antibiotics has already been disproven, and our job now is to find through what unlikely mechanisms these unlikely compounds act.

The area to investigate for clues to the mode of action of antibiotics is obviously the subtle differences that do exist between bacterial and host cells. The explanation of the mode of action of an antibiotic must always include an explanation of why the particular 'reaction is specific for the bacteria and does not affect the host cell.

Such a proposal has been made for the mode of action of penicillin. Since penicillin apparently prevents the formation of the cell wall, a structure peculiar to and essential for bacterial survival, it is easy to see why host cells remain immune to the drug while the bacterial population in the same milieu is rapidly destroyed.

I would like to propose and offer some evidence for another mechanism whereby streptomycin may destroy bacteria without damaging host cells, even though the drug in question might be strongly inhibitory for metabolic reactions in both cellular types.

The proposal states that a streptomycin-induced protein synthesis must precede killing of the cell by streptomycin. The reverse statement obviously is that cells lacking the potential for this induced protein synthesis cannot be killed by this antibiotic. Presumably the latter type cells would include both host cells and bacterial mutants which have developed resistance to the drug. The origin of this hypothesis goes back to our work of some years ago [1] in which we found that aerated, resting suspensions of Escherichia coli were protected by inorganic phosphate against loss of viability resulting from exposure to streptomycin. The lethal effect of streptomycin was found to be inversely proportional to the concentration of inorganic phosphate in the medium. The protective effect of inorganic phosphate did not occur in unaerated suspensions.

One of the interesting aspects of these data is the long period of slow decline in viability before the rapid exponential decline begins. It would, therefore, appear that some precondition must be met before active killing commences.

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*This study was supported in part by National Science Foundation Grant No. G-9891.

The means for studying this phenomenon appeared to us after the interesting finding reported by Anand and Davis [2] that chloramphenicol prevents killing of E. coli by streptomycin when the two drugs are added concomitantly. We had also observed, as they reported, that streptomycin, unlike chloramphenicol, does not cause an immediate block in protein synthesis but always permits some protein synthesis to occur. Since chloramphenicol is a potent inhibitor of protein synthesis in bacterial systems, it occurred to us that the drug might act by inhibiting synthesis of an inducible protein required for the later lethal action of streptomycin. The decreased rate of killing during the first hour in the resting cell experiment might likewise be caused by the same requirement.

To test this hypothesis, we performed the following experiment (Table 1).

Table 1. Effect of Prior Exposure to Stretpomycin on Subsequent Killing of Escherichia coli

Viable count, cells/ml, in presence of 10 µg/ml of streptomycin		Viable count after 2 additional hours in chloramphenicol plus streptomycin			
		Concentration of streptomycin			
Zero time	After 60 min	Zero	10 µg/ml	20 µg/ml	40 µg/ml
$1.7 \cdot 10^7$	$4.5 \cdot 10^6$	$2.2 \cdot 10^6$	$6.7 \cdot 10^5$	$2.8 \cdot 10^4$	$8.7 \cdot 10^3$

Resting cells, suspended in saline and lactate, were subjected to 10 µg/ml streptomycin for 60 min. The viable count decreased from $1.7 \cdot 10^7$ to $4.5 \cdot 10^6$ cells/ml. Chloramphenicol was then added and the cells were spun down and washed in chloramphenicol solution to remove any residual streptomycin. Aliquots were then suspended in a mixture of chloramphenicol and varying concentrations of streptomycin. We found rate of killing to be a function of the external concentration of streptomycin in the presence of chloramphenicol, providing the cells received a prior exposure to streptomycin in the absence of chloramphenicol. If the cells are exposed to chloramphenicol before addition of streptomycin this effect is not observed.

In the next experiment (Fig. 1), E. coli resting cells ($4 \cdot 10^7$/ml) suspended in 1% saline and 0.011 M lactate, at pH 7.0, were exposed to various concentra-

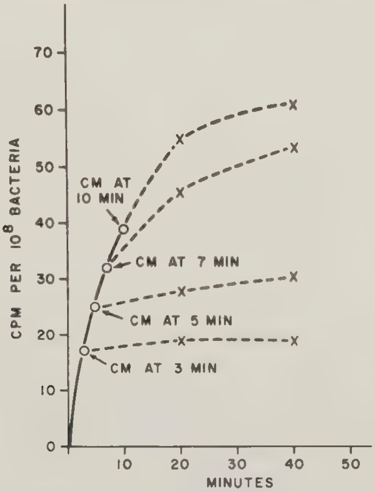

Fig. 1. Effect of varying concentrations of streptomycin during the induction phase on bactericidal sensitivity.

tions of streptomycin for 60 min. Chloramphenicol (30 μg/ml) was then added, the cells were spun down, washed, and resuspended in the same medium plus 30 μg/ml chloramphenicol and 10 μg/ml streptomycin. Viable counts, as before, were made on nutrient agar in quadruplicate.

The bactericidal sensitivity by streptomycin in the presence of chloramphenicol was increased by the concentration of streptomycin during the prior exposure.

We were fortunate in obtaining a supply of C^{14}-labeled streptomycin through the generosity of Dr. Rosenblum, of Merck, Sharpe, and Dohme, which permitted us to carry out labeling experiments.

In these experiments (Fig. 2) we used E. coli growing exponentially in nutrient broth, using the technique described previously by Szybalski and Mashima [3]. An overnight culture was inoculated into a flask of nutrient broth and incubated under aeration until the cell reproduction was exponential according to nephelometric readings, and a cell density of about $2 \cdot 10^8$ cells/ml. Streptomycin (50 μg/ml, specific activity 0.032 μC/μmole) was added at zero time while chloramphenicol (30 μg/ml) was added 3, 5, 7, and 10 min after the addition of labeled streptomycin. Aliquots were taken at indicated times for plating on S + S membrane filters (20-mm diameter). After washing with nonlabeled streptomycin solution and air drying, the C^{14} uptake of the bacteria (about $5 \cdot 10^8$ per filter) was measured in a gas flow counter with a Mylar window having a counting efficiency of 11%.

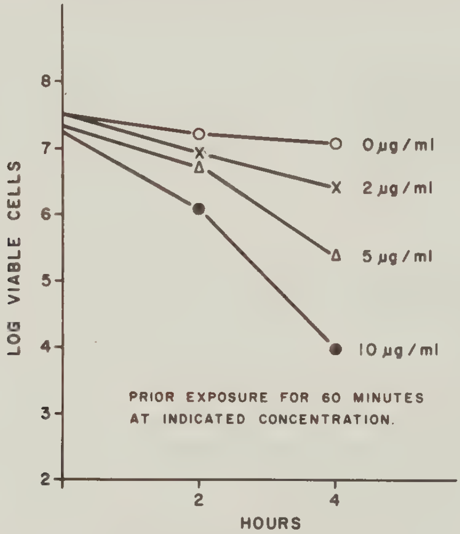

Fig. 2. Effect of prior exposure to streptomycin on the rate of accumulation of C^{14}-label from streptomycin in the presence of chloramphenicol.

When chloramphenicol was added 3 min after the addition of streptomycin, uptake of C^{14}-streptomycin abruptly stopped. The longer the chloramphenicol addition was delayed the greater was the subsequent rate of incorporation of C^{14}-streptomycin into the cells. After 10 min, addition of chloramphenicol had no further effect on the rate of C^{14} incorporation.

Assuming that chloramphenicol acts specifically on protein synthesis, it would appear that some protein synthesis must precede killing by streptomycin. Furthermore, this protein synthesis must be induced by streptomycin itself. The results, therefore, indicate that killing by streptomycin can be divided into two phases, an induction phase which is chloramphenicol-sensitive, and a killing phase which is chloramphenicol-insensitive. In order for streptomycin-killing to occur, the induction phase must precede the chloramphenicol-insensitive phase. If the induction phase is blocked by chloramphenicol, the lethal effect of streptomycin is prevented.

The rate of killing is proportional to the external streptomycin concentration in both the induction and killing phases. This indicates that once a given amount of induced protein is synthesized, the rate of killing is proportional to the external streptomycin concentration. It also indicates that the rate of synthesis of the induced protein is proportional to the streptomycin concentration.

The experiment in which the incorporation rate of labeled streptomycin was measured as a function of the extent of exposure to streptomycin before addition of chloramphenicol (Fig. 2) supports the above conclusions. The longer the induction phase is extended the more rapid is the rate of accumulation of radioactive material in the bacteria.

The data establish the prerequisite for a streptomycin-induced, chloramphenicol-sensitive protein synthesis for killing of bacteria by streptomycin.

Less well established, but suggested by the accumulation of C^{14}-label, is the possibility that the induced protein may be a specific transport system (permease) which facilitates entry of streptomycin into sensitive, i.e., inducible bacteria. According to this hypothesis, resistant mutants would be bacteria which had lost the ability to synthesize the transport system and therefore could not mobilize sufficient streptomycin at the internal sensitive sites for killing to occur. Host cells would also be immune to streptomycin because of their lack of potential to synthesize the transport system.

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DISCUSSION

DR. HAHN: Dr. Hurwitz, assuming that your hypothesis is correct, you should be able to prevent killing of bacteria by streptomycin in a situation in which an amino acid auxotroph is deprived of its essential amino acid. You should also be able to stop the killing process as well upon restoring the essential metabolide to the system. Have you done this experiment?

DR. HURWITZ: No, we haven't done that experiment yet, although I think that in Davis' laboratory they have already shown, using amino acid deficient mutants, that in the case in which you deprive the bacteria of its required amino acid there is no killing similar to the case of chloramphenicol. The rest of the experiment has not been done, however, it should be. I could predict what would happen.

DR. LANTIN (Dietrich): I wonder whether the evidence for the hypothesis expressed by Dr. Hurwitz, that streptomycin induces an enzyme, is conclusive and I am going to propose a viewpoint which I think brings Dr. Hurwitz's results somewhat in closer consonance to those reported by Dr. Brock and also by Anand and Davis. In particular, I wonder whether one might not be able to say that the protein synthesis which is inhibited by the streptomycin and which is necessary for streptomycin killing is a synthesis of cell membrane. If chloramphenicol stops any further synthesis of the cell membrane, streptomycin killing ceases and whatever damage has been done to the cell membrane prior to the addition of chloramphenicol has its effect. It seems to me that this interpretation of a protein synthesis involved in streptomycin killing is consistent with Dr. Hurwitz' results. I may be mistaken—and possibly Dr. Hurwitz could enlarge on this.

DR. HURWITZ: This, of course, is the proposal that was put forward by the Harvard group. It seems to me, if killing occurred as a result of leakage of intermediates from the cell, as they have proposed and as the speaker here proposes here, too, that certain things would have to follow. I think the results of the second slide are not consistent with such a hypothesis. You see, if streptomycin blocks formation of a cellular membrane or results in the formation of a cellular membrane which is faulty, that is, which has lost its selective capacity for retaining intermediates in the cell, we could think of this as a membrane that has holes in it, so that intermediates could leak out the cell. At the time the chloramphenicol was added, any further change in the membrane would have ceased because no more membrane, presumably, could be formed. This hypothesis implies that chloramphenicol can block formation of such a faulty membrane. We would then arrive at a situation at the time of addition of chloramphenicol in which, if there were sufficient holes for enough of the intermediate to leak out of the cell, the cell would then die, presumably as a result of starvation. If there had not been sufficient holes produced at that time, then the cells would continue to live. But certainly, the following can be said, that after the chloramphenicol is added, since no further changes could occur, the presence of streptomycin in the medium should have no further effect on killing. And yet we see from the data that the killing is now a function of the external streptomycin concentration in the presence of chloramphenicol. Actually, the amount of excretion that the Harvard group has reported, is really not enough to account for killing of the cells. Under the conditions of our resting cell experiments we reported excretion of nucleotides and found only a minimal amount of decrease in viability. We had enormous amounts of nucleotides that were excreted into the medium, and yet this did not kill the cells. Many other workers have demonstrated a similar phenomenon, especially with resting cells. Even with growing cells, under certain conditions, you can get quite a spill-out of intermediates and this does not cause death of the cells. I do not think that the amount of leakage of intermediates that they have demonstrated is sufficient to account for killing, nor do I think that such a proposal is consistent with our own results. I do not see how the streptomycin concentration in the presence of chloramphenicol could provide for killing of the cells.

DR. BROCK: In my own work with streptococci, it would seem to me that I found two effects of streptomycin. One at higher concentrations would seem to be a more general disruption of a number of processes while at the lower concentrations growth is inhibited, but then protein synthesis was not. And I could visualize that an antibiotic like streptomycin might have different effects, depending upon the concentration at which it was used in a particular system and the conditions under which the cells are used in the experiment. Dr. Davis' work has been, I think, primarily or exclusively with growing cells, whereas Dr. Hurwitz used what would have to be called, I suppose, nongrowing cells, since they are in phosphate buffer. And it seems to me that that may account for some differences.

Secondly, I don't believe that Davis has ever proposed, and I surely wouldn't want to propose, that death is due to the mere loss of constituents from the cell, because if this were true, you would think that you could put these cells on a complex medium containing all the usual growth factors and they would be able to revive. But, of course, viable counts are easily done on complex agars, so this apparently is not true. The leakage is undoubtedly a secondary effect and I believe that the important thing in all of these studies is to distinguish between a primary effect and a secondary effect. The experiment that extends over a period of 4 or 5 hr seems to me to be open to so many criticisms from the point of view that a number of the observations were secondary, that I, frankly, at this stage of our studies of modes of action of antibiotics, do not get excited about any effect that was not shown at least in the first 30 min to 1 hr and preferably in the first 5 min after the antibiotic was added. This seems to me to be essential if we are going to get to the primary site of attack. There are so many secondary things that can occur later that might confuse the issue and I would plead for this sort of experiment that is very short term, and this, of course, involves the use of isotopes to get any effect in a very short period of time.

DR. STROMINGER: I'd like to ask Dr. Brock if iso-novobiocin induces beta-galactosidase activity in his strain. The reason I ask that question is because Dr. Brock mentioned that we had shown that novobiocin will induce uridine nucleotide accumulation in Staphylococcus aureus. Much to our surprise, so does iso-novobiocin, although I must also say that our sample of iso-novobiocin was a rather old one and since the isomerization occurred under rather mild conditions, we are not sure that the sample of iso-novobiocin we used was a pure sample.

DR. BROCK: This is one of the first things I wanted to try and so I called Upjohn and asked them to send me some. They said they would and three days later I got a letter saying that there was no more available, so apparently it was all out at St. Louis. I would like to try this experiment, but I could not get any iso-novobiocin to do so. I've been thinking about making some myself. It's a relatively easy thing. It's an alkaline isomerization, but I haven't gotten around to doing this. As you say, there may be a problem about purity in this regard.

DR. SAZ: Dr. Brock, I should like to ask if novobiocin has any effect so far as you know on mannose utilization or metabolism.

DR. BROCK: I haven't tried mannose. I used glucose because I had it available and because in most systems that I have seen glucose and mannose are pretty much in equilibrium and glucose goes to mannose quite readily. Further, Gilbey and coworkers have shown radioactive glucose did go into the mannose of the membrane. It's one of the experiments that I have in mind for the future.

G. PETERSON (Houston, Texas): This is in the form of a comment rather than a question. There is evidence in the literature for streptomycin induction of some protein synthesis, strangely enough, polyphenolase in potatoes, whatever connection that may have with these reports today. I was wondering if anyone has ever tried this. I don't know of any attempts to repeat this sort of experiment, but it seems to be very a propos to some of the reports this morning.

DR. SAZ: Does anyone know anything about this?

DR. PETERSON: This reference was in Science about two years ago.

DAVID WOLF (Walter Reed): Dr. Hurwitz, I was wondering if you had attempted to measure any amino acid incorporation for any protein synthesis in that first hour in your experiments. I know it's rather difficult to pick up any enzyme synthesis this way, but in addition to this question, if chloramphenicol had been added in the first hour, what do you think the effect would have been?

DR. HURWITZ: You mean what would have been the effect of chloramphenicol on the labeled amino acid incorporation in the first hour in the arrested cell suspension?

DR. WOLF: Well, two parts. First of all, did you ever attempt to measure any amino acid incorporation; and second, have you tried chloramphenicol in the resting experiment in the first hour?

DR. HURWITZ: No, we have not tried measuring amino acid incorporation in the resting cell suspension. All of our work on amino acid incorporation and the effects of streptomycin on various macro-molecule synthesis has been in penicillin induced spheroplasts, mainly because the amount of incorporation in arrested cell suspensions would be pretty low and difficult to measure. These experiments will have to be done, I think, in order to document our thesis that a protein synthesis actually occurs. I think this is a valid comment and we are going to do this, but there are only so many hours in a day.

INHIBITION OF PROTEIN SYNTHESIS BY ANTIBIOTICS

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Studies on the modes of action of antibiotics are not only of interest to active workers in this field or to those concerned with theoretical problems of chemotherapy. Investigations of the mechanisms of inhibition of microbial growth necessarily lead into studies on the basis of growth and identical reproduction. This topic represents one of the central themes of the biological sciences today.

One general key to the solution of problems of antibiotic modes of action is perhaps provided by the criteria according to which antibiotics are selected. Antibiotics emerge from empirical screening and testing experiments as substances that exhibit a high degree of selective toxicity, i.e., they are highly toxic to microorganisms but, in contrast, show low toxicity for mammalian organisms and particularly for man.

This criterion eliminates from consideration as targets of antibiotic action those processes that occur identically in microbial and mammalian cells and, with stronger reason, processes that occur only in multicellular mammalian organisms but not in unicellular microorganisms. Therefore, selective toxicity for microbes directs one's attention to processes that are specific to the physiology of microorganisms but do not occur in mammalian cells or tissues.

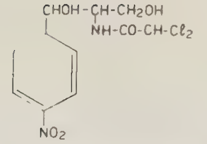
One lucid example of antibiotic action directed against a specific microbial process is the interference by penicillin and cycloserine with the biosynthesis of a certain peptide that is a constituent of bacterial cell walls [1]. Mammalian cells do not possess the architectural and chemical equivalents of such walls nor do they have the chemical machinery for the biosynthesis of specific wall components.

Not all antibiotic actions, however, are directed against processes that are specific for microbial cells. In this presentation we are concerned with a more difficult situation, namely, the effects of certain antibiotics on protein synthesis. Protein biosynthesis is not peculiar to microorganisms; it also occurs in mammalian cells and, indeed, in all known life forms. It appears to be the key process by which genetic constitution is phenotypically expressed.

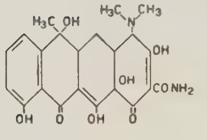
The reasons why certain antibiotics inhibit protein synthesis selectively in microbial cells but not in mammalian cells are still quite unknown. One must surmise that microbial protein synthesis differs in some mechanistic detail from mammalian protein synthesis and that this very difference provides the basis for the selective toxicity of certain antibiotics.

The problem of the mechanism of antibiotic action upon protein biosynthesis is further complicated by the fact that a variety of structurally unrelated antibiotic substances produce the same general effect (Fig. 1).

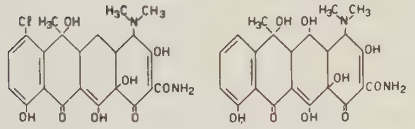
Chloramphenicol (chloromycetin) and chlortetracycline were the first antibiotics that were found to inhibit bacterial protein synthesis. Despite much intellectual effort we have been unable to recognize structural similarities between the molecules of chloramphenicol and of tetracyclines which might be responsible for the similarities in the effects of these antibiotics upon protein synthesis.



Chloromycetin



Tetracycline (Achromycin)



Chlorotetracycline (Aureomycin) Oxytetracycline (Terramycin)

Fig. 1.

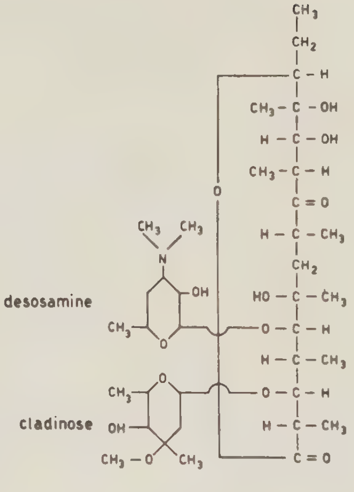


Fig. 2. Erythromycin.

Brock and Brock [2] have shown recently that the action of erythromycin (Fig. 2) on bacterial protein synthesis is very similar to that exhibited by chloramphenicol. Erythromycin consists of a macrolide ring with two unusual sugars substituted in glycosidic linkage. This structure is not reminiscent of the molecules of chloramphenicol or tetracycline nor does it bear a relationship to amino acids, proteins, or nucleic acids. Finally, we have discovered [3] that streptomycin also inhibits bacterial protein synthesis rather specifically. Again, there appears to exist no meaningful structural resemblance between the molecule of streptomycin and the structures of other antibiotic inhibitors of protein synthesis. We are aware, however, that streptomycin contains a guanidino group which is also present in the amino acid, arginine. It is worthy of consideration that this common structural feature may have functional significance.

During the years 1948-1951 it was found that streptomycin [4], chlortetracycline [5, 6], and chloramphenicol [6] inhibit the induced synthesis of enzymes in bacteria. Escherichia coli, growing in a medium containing lactose, synthesizes the enzymes required for the dissimilation of lactose. Consequently, a washed suspension of these bacteria oxidizes lactose at a high rate. This oxidation is not influenced significantly by chloramphenicol (Fig. 3). On the other hand, when the same bacteria are grown in the absence of lactose and lactose is then added to a washed suspension, one observes the induced synthesis of the enzymes necessary to metabolize lactose. This is indicated by an exponential increase in the oxidation rate. Chloramphenicol inhibits the induced synthesis of these enzymes. Analogous results are obtained with chlortetracycline and have been extended to the induced synthesis of enzymes that are required for the oxidation of maltose, arabinose, and acetate [6].

It can be shown that chloramphenicol interferes with the actual synthesis of enzyme proteins rather than with the primary process of induction (Fig. 4). Addition of chloramphenicol at time intervals to a culture in series that is actively synthesizing enzymes, immediately inhibits the formation of additional enzyme, and the formerly exponential reaction rates thus become linear [6]. Finally, these antibiotics cannot act by inhibiting induced enzyme formation because these substances inhibit bacterial growth in organic media when no

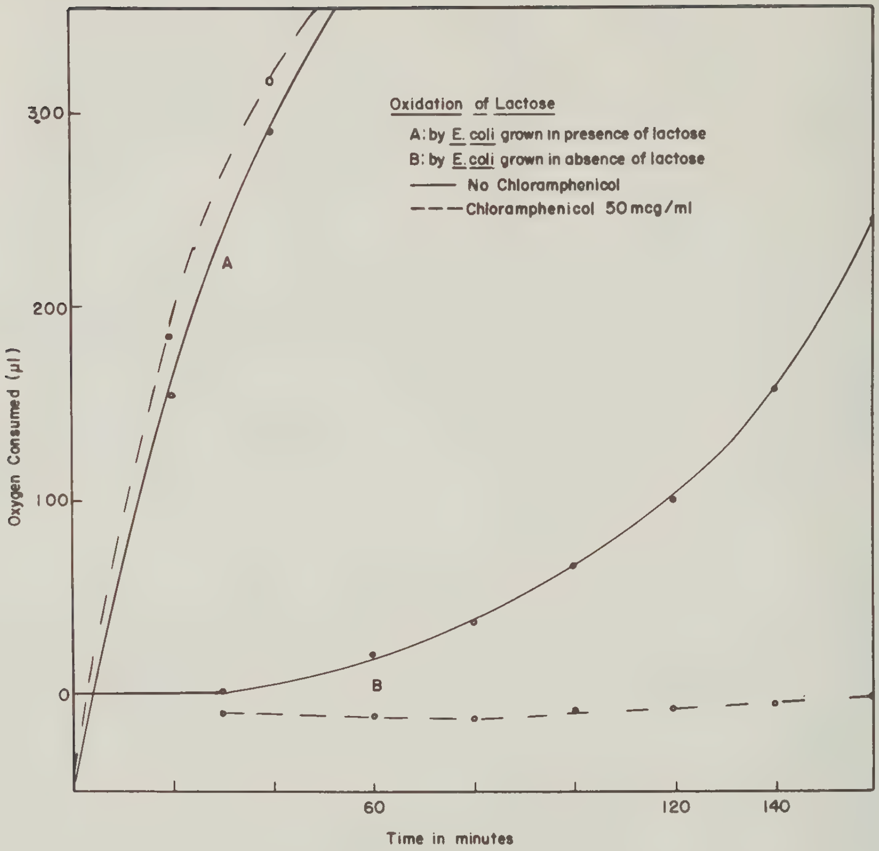


Fig. 3. Chloramphenicol inhibition of adaptation in E. coli for the oxidation of lactose and the failure of chloramphenicol to influence lactose oxidation in previously adapted organisms.

induced enzyme synthesis is required. We have interpreted the actions of antibiotics on induced enzyme synthesis as indications of a more general effect, namely, an interference with bacterial protein synthesis [6].

This idea is verified by experimental results of the type depicted in Fig. 5. Addition of chloramphenicol to a culture of E. coli in the logarithmic phase of multiplication results in a nearly immediate arrest of protein synthesis. Other biosynthetic processes, however, and notably the synthesis of both types of nucleic acid continue at linear rates over several generation times [7, 8]. Identical results for E. coli were reported from the laboratory of Gale by Harrington [9] with chloramphenicol, for Staphylococcus aureus by Gale and Folkes with chloramphenicol and chlortetracycline [10], and for E. coli by Brock and Brock with erythromycin [2]. Especially with chloramphenicol, the production of excess nucleic acids in the absence of protein synthesis can occur without appreciable loss of viability in the experimental cultures [8]. The nature and properties of these surplus nucleic acids continue to be an interesting field of study in this and other laboratories.

A somewhat similar situation was encountered in experiments with streptomycin [3]. Addition of this antibiotic to exponentially dividing cultures of E. coli produced complete inhibition of protein synthesis within a fraction of one generation time, whereas the formation of substances absorbing light at 260 mµ continued at a decreasing rate (Fig. 6).

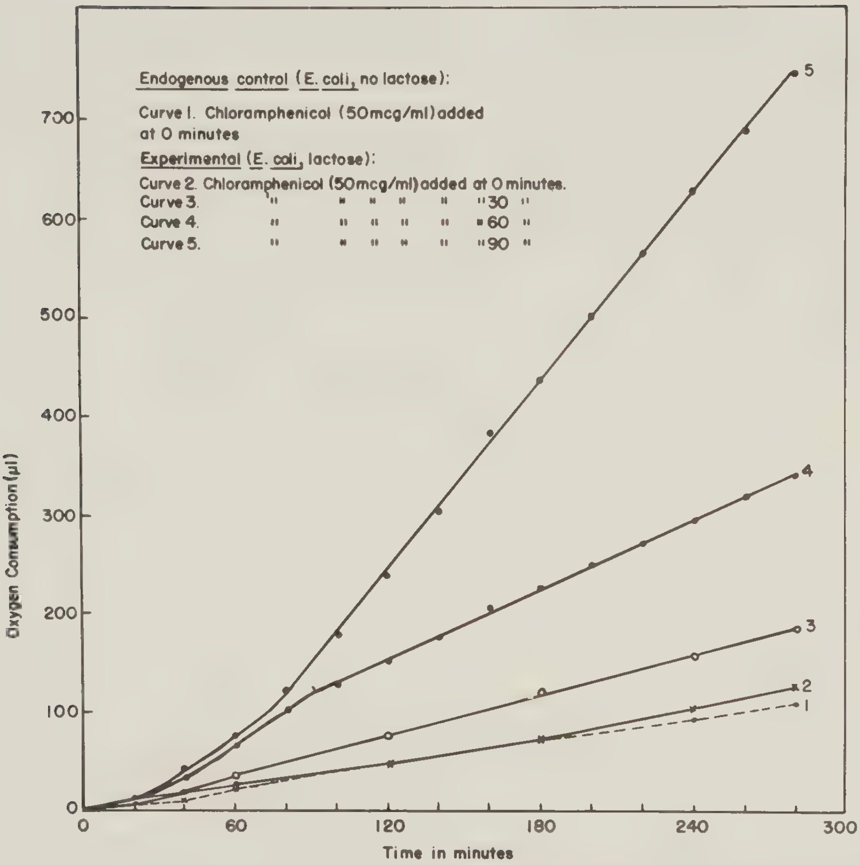


Fig. 4. Arrest of adaptive enzyme formation in E. coli by chloramphenicol addition at intervals during adaptation of lactose.

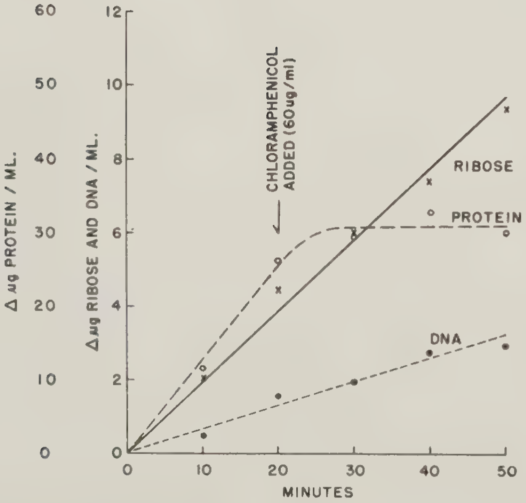


Fig. 5. Chloramphenicol inhibition of protein synthesis in E. coli B with continued nucleic acid formation.

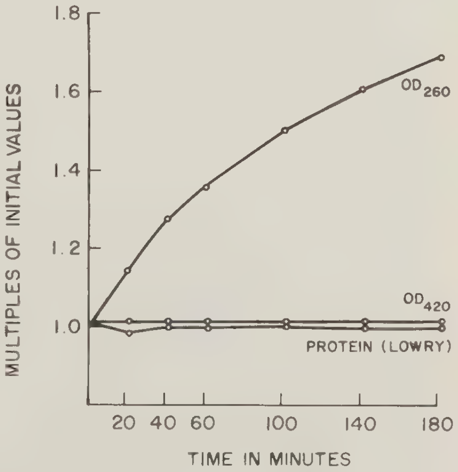


Fig. 6. Streptomycin inhibition of protein synthesis.

Chemical analysis showed that most of this 260-mμ-absorbing material was accumulated as intracellular polynucleotides. Within approximately one generation the quantities of intracellular nucleic acids, both RNA and DNA, increased by approximately 70%. The base composition of bacterial nucleic acid after exposure to streptomycin was identical to that of the bacteria before streptomycin had been added. Any gross difference in the base composition of nucleic acids that were synthesized during streptomycin action would have been detectable even against the analytical background of the preexisting normal bacterial nucleic acids [11]. Table 1 shows analytical data on the base composition of pre- and post-streptomycin nucleic acids.

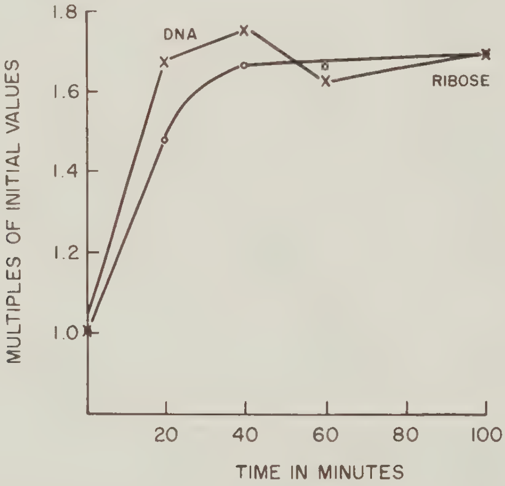


Fig. 7. Nucleic acid synthesis in streptomycin-exposed E. coli.

Table 1. RNA Composition of E. coli, Strain Gratia, in Moles Specific Nucleotide/100 Moles Total Nucleotide in RNA

Organism	Adenine	Guanine	Cytidylic acid	Uridylic acid	Molar ratios Purines/pyrimidines	6-Am/6-K
Control	26.3	24.8	23.8	25.0	1.05	1.01
Streptomycin-exposed	26.3	22.1	26.9	24.7	0.94	1.13

DNA Composition of E. coli, Strain Gratia, in Moles Specific Nucleotide/100 Moles Total Nucleotide in DNA

Organism	Adenine	Guanine	Cytosine	Thymine	Molar ratio, A+T/G+C
Control	23.5	23.5	27.2	25.7	0.97
Streptomycin-exposed	24.0	22.8	27.3	26.0	1.00

We are thus dealing with two different types of action of antibiotics on bacterial protein synthesis. The first is exhibited by chloramphenicol, chlortetracycline, and erythromycin. These agents are predominantly bacteriostatic and nucleic acid synthesis is not affected by them for some time. The other is exhibited by streptomycin which is markedly bactericidal, and under whose influence nucleic acid synthesis continues for little longer than one generation and then ceases. It should be pointed out that in streptomycin action the decrease in the rate of nucleic acid formation is much slower than the rate at which viability is lost so that one measures largely nucleic acid biosynthesis in cells that have lost their ability to form colonies on plates.

The actions of chloramphenicol and of tetracyclines on bacterial growth are additive [12]. Table 2 lists concentrations of chloramphenicol and oxytetracycline alone or in combinations that produce 50% reductions in the growth rate of E. coli.

Table 2. Concentrations of Chloramphenicol and Oxytetracycline in Combinations Producing 50% Growth Inhibition of E. coli

Chloramphenicol		Oxytetracycline		Sum of fractional inhibitory concentrations
Concentration, μg/ml	Fractional inhibitory concentrations	Concentration, μg/ml	Fractional inhibitory concentrations	
0	0	0.17	1.00	1.00
0.06	0.07	0.16	0.94	1.01
0.12	0.14	0.16	0.94	1.08
0.25	0.29	0.13	0.76	1.05
0.50	0.57	0.09	0.52	1.09
0.80	0.92	0.02	0.12	1.04
0.87	1.00	0	0	1.00

From these data we have calculated the fractional inhibitory concentrations for each antibiotic in the pairs. The last column lists the sums of these fractional inhibitory concentration which in all instances are close to unity. Similar results were obtained with chloramphenicol and chlortetracycline or with the two tetracyclines. These results prove that under the conditions of these experiments the actions of the three antibiotics in all possible paired combinations were additive. We interpret this finding as circumstantial evidence that their modes of action are related in some manner. Perhaps we are dealing with a situation of concurrent blocking of different synthetic pathways that jointly contribute to the same end result, namely, protein biosynthesis.

In order to discuss inhibitory effects upon protein synthesis, it is necessary to summarize briefly the current state of thinking concerning the reaction sequence in protein biosynthesis. The first step is apparently an integrated reaction of amino acid activation, dependent upon ATP, and the synthesis of amino-acyl—low-molecular RNA. The enzymes that catalyze these reactions are being referred to as "activating enzymes" or better as amino-acyl—RNA-synthetases. There seems to exist one specific enzyme and one specific low molecular RNA (molecular weight in the order of 30,000) for each of the approximately 20 amino acids.

The next step is the integration of amino acids into protein molecules with the aid of the cytoplasmic ribosomal particles. These particles consist of high molecular RNA and protein, and perhaps contain or represent the templates that are believed to direct the establishment of the specific amino acid sequences which determine the specificities of proteins. This integration step requires GTP, reduced glutathione, and an enzyme fraction which is perhaps less heterogenous than the group of amino-acyl—RNA-synthetases that catalyze the first step.

Ultimately, finished proteins will have to separate from the site of their formation and fold into their specific secondary structures upon which, for example, enzymatic activities depend.

The question now arises as to where in this reaction sequence the different antibiotic inhibitors of protein synthesis interfere. Information on this point is incomplete and inconclusive. The antibiotic most studied is chloramphenicol. DeMoss and Novelli [13] and later Lacks and Gros [14] have shown that amino acid activation and synthesis of amino-acyl—RNA are not inhibited by chloramphenicol. The incorporation of amino acids into bacterial ribosomal particles, however, is inhibited by chloramphenicol as shown by Lamborg and Zamecnik [15]; to a lesser extent, this inhibition is also observed in experimental systems from higher plants [16] which produce net synthesis of soluble proteins. In an experimental system of mammalian origin, chloramphenicol does not inhibit the incorporation of amino acids into microsomes [17].

Chloramphenicol appears to be active somewhere in the sequence of reactions which transfer amino acids from combination with low-molecular RNA to peptide linkage in proteins. This step may be sufficiently different in bacterial and in mammalian systems to account for the selective toxicity of chloramphenicol in the microbial systems.

We are unaware, at this time, of any published data that are concerned with the actions of tetracyclines or of erythromycin upon the individual reaction steps in protein synthesis. For streptomycin, Erdos and Ullmann [18, 19] have suggested an interference with the transfer of amino acids from low-molecular RNA to protein, a type of action similar to that of chloramphenicol. A complication of the streptomycin problem is introduced by observations [20, 21, 22] that chloramphenicol is capable of protecting bacteria from the bactericidal action of streptomycin. We have no ready explanation for these observations except to assume that inhibition of protein synthesis is not the primary action of streptomycin but rather one of its consequences.

Two areas of experimental findings concerning bacterial protein synthesis and the action of chloramphenicol upon this process are difficult to integrate into a consistent train of thought.

First, Beljanski and Ochoa [23] have obtained indications of protein synthesis in disrupted bacterial systems that required a fraction consisting of fragmented bacterial membranes, but did not appear to depend upon the amino acid activation step mentioned earlier. Not only did these systems incorporate radioactive amino acids into proteins but they actually produced small net increases in protein, demonstrating actual protein biosynthesis. These reports [23], kinetic studies by Lacks and Gros [14] and studies on activating enzymes by Novelli [24], have cast some doubt on the exclusive nature of the protein synthesizing reactions involving activating enzymes, low-molecular RNA, and ribosomal particles as reactants. Membrane-dependent protein synthesis is also inhibited by chloramphenicol [23] although as little is known about the underlying mechanism as in the case of ribosomal protein synthesis.

It is not possible to resolve this issue by discussion at this time. Perhaps chloramphenicol is an inhibitor of certain categories of peptide bond synthesis such as that occurring in protein synthesis, while it fails to inhibit peptide bond formation of the types which occur in glutathione synthesis [25] or in the formation of bacterial cell wall peptides [26].

Secondly, we must concern ourselves with the action of one of the three stereoisomers of chloramphenicol upon bacterial biosynthesis of glutamyl polypeptide (Fig. 8). Marked growth inhibitory properties reside only in the D-threo isomer which is the natural drug. The L-erythro isomer exhibits only very slight antibiotic activity [27], and the two remaining isomers do not inhibit bacterial growth. (Unless otherwise modified, chloramphenicol should be taken to mean the D-threo isomer.)

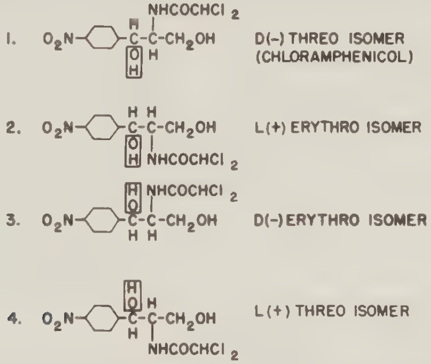


Fig. 8. Chloramphenicol and its stereoisomers.

Since proteins whose formation is inhibited by a D-isomer are composed of amino acids of the stereochemical L-series, it was considered possible that an antipodal relationship might exist between the stereochemical configuration of the inhibitor and that of the building blocks of proteins. If this were the case, one should expect that the formation of an amino acid polymer, made of D-amino acids, should be inhibited by one of the L-isomers of chloramphenicol through an analogous but stereochemically converse mechanism of action.

A broken line on the left side of the diagram (Fig. 9) represents a growth curve of Bacillus subtilis. The solid line indicates how this growing culture produced relatively large quantities of extracellular D-glutamyl polypeptide. The right side of the diagram shows that the L-erythro isomer of chloramphenicol

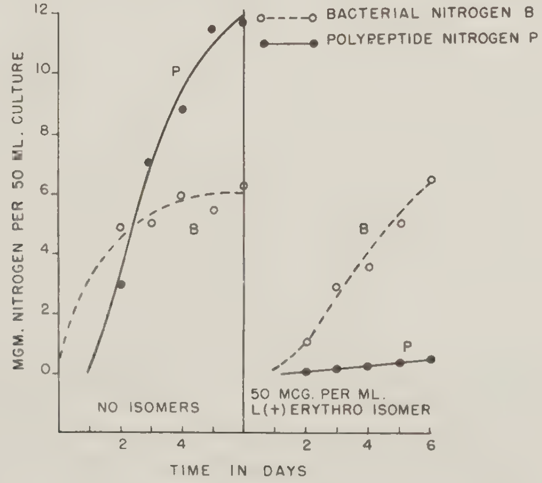


Fig. 9. B. subtilis. Growth and polypeptide formation.

inhibited the biosynthesis of glutamyl polypeptide but did not inhibit growth and, thus, protein synthesis of the bacilli [28].

When the surface growth of B. subtilis, after synthesizing polypeptide for a number of days, was floated on fresh medium, the bacilli continued to produce polypeptide. The bacilli also continued to produce polypeptide in the presence of the antibiotic, chloramphenicol, while the L-erythro isomer inhibited the formation of polyglutamic acid as before (Fig. 10).

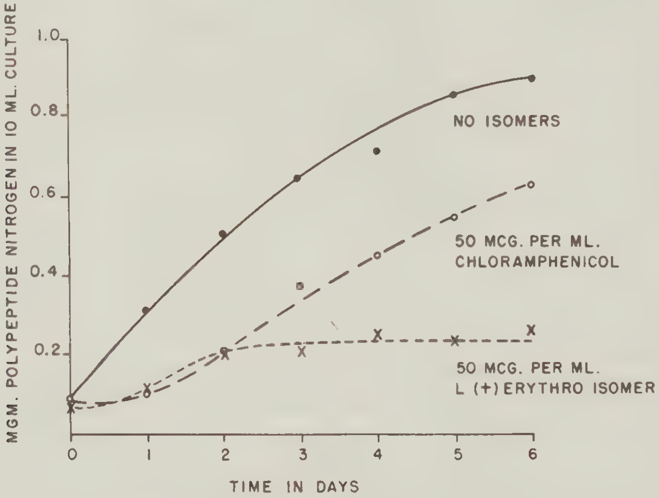


Fig. 10. B. subtilis. Polypeptide formation by full-grown cultures.

These findings can be summarized as follows: the L-erythro isomer inhibited the formation of D-glutamyl polypeptide but did not affect growth and, therefore, protein synthesis; the D-threo isomer, chloramphenicol, inhibited growth of the bacilli but did not interfere with the formation of glutamyl polypeptide. The remaining two isomers were nonactive [28].

Assuming that the actions of chloramphenicol on protein synthesis and of the L-erythro isomer on polyglutamate synthesis are based on analogous mechanisms of action, it may be surmised that they affect some phase in the condensation of amino acids to polymers rather than the assemblage of amino acids into specific sequence patterns.

The present state of problems of antibiotic action upon protein biosynthesis is such that their solution will depend upon further advances on the knowledge of the mechanistic details of this synthetic process. In this area of study one must also expect to find explanations for the selective toxicity of antibiotics that inhibit specifically microbial protein synthesis.

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INHIBITION BY PUROMYCIN OF AMINO ACID INCORPORATION INTO PROTEIN*

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More than one hundred years ago the French physiologist Claude Bernard recognized the value of poisons as "vital reagents." His studies of the arrow poison curare and of carbon monoxide stemmed, not from an interest in their toxicology, but from an appreciation of their usefulness as instruments of dissection, instruments which facilitate understanding of "the most delicate phenomena of the living organism." Since the time of Claude Bernard a large number of antimicrobial agents have been recognized in medicine. Their value as biochemical tools is only beginning to be appreciated in part through the efforts of our moderator and members of this panel.

The work about which I have been asked to talk was undertaken with the aim of studying protein synthesis by subjecting an in vitro system capable of incorporating amino acids into protein to the action of poisons which appeared a priori likely to block the process of amino acid condensation. The two agents we chose to investigate were di-isopropylfluorophosphate, an inhibitor of several enzymatic trans-peptidation and trans-esterification reactions [1], and puromycin, an inhibitor of induced enzyme synthesis [2]. Of these two reagents only puromycin appeared to be effective at low concentrations and its effect was therefore investigated further. A full account of this work appears elsewhere [3].

Puromycin is a broad-spectrum antibiotic produced by Streptomyces alboniger [4]. Its structure, proposed first in 1953 [5,6], was shortly confirmed by total synthesis [7]. It consists of a purine linked by a β-glycosidic bond to an aminosugar linked, in turn, by an amide bond to an amino acid (Fig. 1a). Hydrolysis of the molecule at the β-glycosidic linkage cleaves the molecule into two biologically inactive fragments [5]. On the other hand, the aminonucleoside, i.e., the portion remaining after splitting off the amino acid, and a number of derivatives in which various amino acids are substituted for the o-methyl-L-tyrosine of puromycin [8] do possess activity against protozoan and mammalian cells [9, 10].

The growth and viability of cells from a variety of organisms, from microorganisms to mammals, are diminished or abolished by puromycin, or its aminonucleoside [4,10-14,16-20]. Reversal of these effects by purines or purine nucleotides has been demonstrated in certain cases [16-20], but the specificity of these reversals is confusing. Apart from such results, which have been taken to suggest that puromycin might interfere with nucleic acid metabolism [20], two considerations point to the possibility that puromycin is a specific inhibitor of protein synthesis. First, the minimum bacteriostatic concentration

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*This work was supported by grants from the National Institutes of Health, U. S. Public Health Service, and the American Cancer Society.

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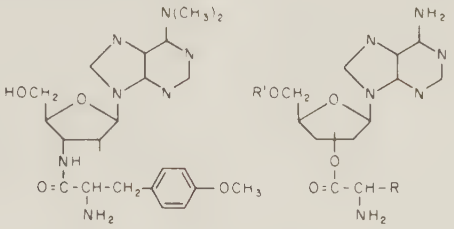
‡Contribution No. 340 of the McCollum-Pratt Institute.

of puromycin for *Staphylococcus aureus* is sufficient to completely block the induced synthesis of β-galactosidase in this organism [2]. Secondly, puromycin is a structural analog of the amino-acid bearing end of the transfer ribonucleic acids [21, 22, 23] which are apparently ubiquitous intermediates in the biosynthesis of protein (Fig. 1). It would therefore appear that puromycin might inhibit protein synthesis at a site involving transfer RNA. A group at Osaka University in Japan [24] have recently examined the in vivo effect of puromycin on the synthesis of protein and nucleic acid by *Pseudomonas fluorescens*. Their findings conform to the expectation that protein synthesis alone is specifically inhibited.



Puromycin Transfer RNA amino-acid

Fig. 1. Structures of puromycin and the amino acid-bearing end of transfer RNA. R represents the remainder of the amino acid residue, R' represents the remainder of the RNA polymer. (Reprinted from Proc. Nat. Acad. Sci., U. S. [3]).

We confined our attention to an in vitro system. A rat liver extract containing microsomes and the soluble enzymes and RNA necessary for amino acid activation and transfer, when incubated together with ATP, a nucleoside triphosphate-generating system, and radioactive leucine, irreversibly incorporates some of the radioactive amino acid into the interior of peptide chains of the protein subsequently isolated [25]. The results of Table 1, obtained with this system, reveal that puromycin at concentrations as low as $5 \cdot 10^{-6}$ M cause a considerable inhibition of the leucine incorporation process. Similar results have since been obtained by Allfrey et al. using isolated calf thymus nuclei incorporating either alanine-1-C^{14}, glycine-1-C^{14} or leucine-1-C^{14} into protein [28]. The observation has also been extended by Bishop et al. [29] to a reticulocyte system, and by Nathans and Lipmann to a system from *Escherichia coli* [15]. It will be noted that when the glycosidic bond of puromycin is cleaved by acid hydrolysis, the resulting fragments, known to be biologically inactive in vivo [5], are found to be inert in vitro.

The incorporation of radioactive amino acid into protein requires the participation of an energy-generating system and is known, through the efforts of a large number of workers, to proceed via three experimentally distinguishable steps* [30].

(1) $\text{AA} + \text{ATP} + \text{E} \rightleftarrows \text{E-AMP-AA} + \text{PP}$

(2) $\text{E-AMP-AA} + \text{sRNA} \rightleftarrows \text{sRNA-AA} + \text{AMP} + \text{E}$

(3) $\text{sRNA-AA} + \text{Ms} \xrightarrow[\text{ATP}]{\text{GTP}} \text{Ms protein} + \text{sRNA}$

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*The following abbreviations are used: s-RNA, soluble ribonucleic acid; AA, amino acid; ATP, adenosine triphosphate; AMP, adenosine-5'-phosphate; AMP-AA, amino acid adenylate; E, amino acid activation and transfer enzyme; PP, pyrophosphate; Ms, microsome; GTP, guanosine triphosphate.

Table 1. Effect of Puromycin on Leucine-C^{14} Incorporation into Protein of a Crude Extract of Rat Liver

Tube number	Puromycin, mμmoles	Counts/min (above background) per mg protein	Inhibition, %
1*	0	0.0	
2	0	27.2	
3	5	14.2	48
4	10	9.0	67
5	20	4.4	84
6	200	0.3	99
7	0	27.3	
8	200†	30.2	0

After 5 min of preincubation at 23 C in the absence of added leucine, incubation with labeled leucine was carried out for 30 min at 37 C in a final volume of 1.2 ml. The incubation mixture contained 0.7 ml crude extract in medium A [26], 1.5 μmoles each of ATP and $MgCl_2$, 20 μmoles of phosphoglycerate, puromycin as indicated, and 0.2 μmoles of DL-leucine-1-C^{14} (0.13 μC L-leucine-1-C^{14} corresponding to approximately $7 \cdot 10^5$ cpm). Tube 7 received 10 μmoles of NaCl.

*Tube 1 received 1 ml of cold 0.73 M trichloracetic acid immediately after addition of tagged leucine.

†Puromycin was hydrolyzed for 20 min in 1 N HCl at 100 C and neutralized with NaOH so that the added solution contributed 10 μmoles of NaCl.

(Reprinted from Proc. Nat. Acad. Sci., U.S. [3].)

In addition to two enzymatic systems for the generation of nucleoside triphosphate, each of the above reactions has been examined for inhibition by puromycin at concentrations of the antibiotic as high as 200 mμ moles/ml. L-leucine was the amino acid employed. Only in the case of reaction (3) was any effect of puromycin observed. Table 2 documents the observation that puromycin inhibits reaction (3) and moreover demonstrates that the extent of this inhibition is sufficient to account for the observed inhibitory effect of puromycin on the over-all reaction represented by the sum of reaction (1), (2), and (3).

The in vivo biological activity of analogs of puromycin containing a variety of amino acid substituents or entirely lacking an amino acid substituent might be explained if puromycin and its derivatives were necessarily hydrolyzed enzymatically to the aminonucleoside which would then serve either as the true inhibitor or its precursor. This hypothesis is rendered unlikely by the experiment shown in Table 3 which states that at equal concentrations of the two substances puromycin is much more effective than its aminonucleoside. This raises the unexpected possibility that the mode of action of puromycin and its aminonucleoside are not identical. This observation may be taken as a warning that in correlating the effect of changes of chemical structure on biological activity the results may be subject to misinterpretation when the biological parameter measured is as gross as viability or growth.

While the experiments reported here do not themselves contribute to our understanding of protein synthesis it should be evident that a deeper analysis of the inhibition by puromycin is likely to afford new insights into the nature of the protein synthetic mechanism. It remains to be determined whether puromycin must be degraded or built into some more complex cellular metabolite before it becomes inhibitory and whether any components of the in vitro system are capable of reversing the effect of puromycin. It is hoped that it will be possible

Table 2. Effect of Puromycin on the Incorporation of Leucine-C^{14} into Protein, Comparing Free Leucine with Leucine Bound to S-RNA

Tube number	Initial form of leucine-C^{14}		Puromycin, mμmoles	Counts/min (above background) per mg protein	Inhibition, %
	Free	Bound to S-RNA			
C 1*	+		0	0.0	
2	+		0	37.7	
3	+		20	13.3	65
4*		+	0	0.0	
5		+	0	19.4	
6		+	20	7.0	64
M 1*		+	0	0.3	
2		+	0	17.1	
3		+	5	14.6	15
4		+	20	9.7	44
5		+	200	2.5	87
6		+	0	14.9	
7		+	20	16.6	0

After 5 min of preincubation at 23 C in the absence of leucine-C^{14}, incubation with the radioactive component was carried out for 30 min at 37 C in a final volume of 1 ml. Each incubation mixture contained: 25 μmoles of potassium phosphate adjusted to pH 7.5, 50 μmoles of sucrose, 0.5 moles each of ATP and GDP, 5 μmoles each of phosphocreatine and $MgCl_2$, and 0.05 ml crude homogenate, those labeled M contained 0.2 ml,microsomal preparation. These extracts were prepared in siotonic (0.25 M) sucrose. Tubes M-1 through M-7 each contained 5 μmoles of unlabeled L-leucine. Where incubation with free leucine-C^{14} is indicated 0.154 moles of DL-leucine-1-C^{14} (0.1 μC L-leucine-1-C^{14} corresponding to approximately $5 \cdot 10^5$ cpm) was present. Where incubation with S-RNA-leucine-U-C^{14} is indicated the material added contributed about 2000 cpm. and contained no more than 0.04 mμmoles of S-RNA-leucine as estimated from its absorbance at 260 mμ, assuming a molecular weight of 30,000 [27] and that one-twentieth of S-RNA is S-RNA-leucine. Puromycin was present as indicated. Tube M-6 recieved 1 μmole of NaCl.

*Tubes C-1, C-4, and M-1 each received 1 ml cold 0.73 M trichloracetic acid immediately after addition of radioactive material.

†Puromycin was hydrolyzed for 20 min in 1 N HCl at 100 C and neutralized with NaOH so that the added solution contributed 1 μmole of NaCl.

(Reprinted from Proc. Nat. Acad. Sci., U.S. [3]).

Table 3. Comparison Between the Effects of Puromycin and Its Aminonucleoside on Leucine-C^{14} Incorporated into Protein of a Crude Extract of Rat Liver

Tube number	Puromycin			Aminonucleoside	
	Antibiotic, mμmoles	Counts/min (above background) per mg protein	Inhibition, %	Counts/min (above background) per mg protein	Inhibition, %
1*	0	2		2	
2	0	371		371	
3	10	284	23	–	–
4	30	126	66	352	5
5	100	33	91	270	27

Incubation was carried out for 10 min at 37 C in a final volume of 1 ml. The incubation mixture contained: 0.45 ml crude extract in medium A [26]. 1 μmole each of ATP and $MgCl_2$, 10 μmoles of phosphocreatin. 0.05 ml of ATP-creatine transphorylase, puromycin or aminonucleoside as indicated, and 0.2 μmoles of L-leucine-U-C^{14} (1.3μC C^{14} corresponding to ca. $7 \cdot 10^6$ cpm).

*Tube 1 received 1 ml 0.73 M trichloracetic acid before addition of rat liver extract.

to synthesize a 2' analog of puromycin from 9-(2-amino-2-deoxy-β-D-ribofuranosyl)-6-dimethylaminopurine [13]. Such an analog could provide evidence concerning which hydroxyl of sRNA is esterified with the amino acid it transfers.

Experiments by Lacks and Gros [32] and by Hopkins [33] and Allfrey et al. [28] suggest that chloramphenicol, like puromycin, inhibits protein synthesis beyond the formation of the sRNA-AA complexes. Aside from the different susceptibilities of animal and bacterial cells to inhibition by chloramphenicol and puromycin a further interesting difference has been noted by Takeda et al. [24]. In P. fluorescens, where, as in other microorganisms [34,35,36], chloramphenicol causes accumulation of a metabolically unstable RNA with different physical properties from the RNA normally present, the RNA which accumulates following puromycin inhibition of protein synthesis appears to be indistinguishable from RNA found in uninhibited cells. This difference between the effects of the two antibiotics suggests that puromycin may be even more selective than chloramphenicol in blocking protein synthesis.

Several antibiotics have been reported here this morning (T. D. Brock) to inhibit the synthesis of microsomal protein by resting cells of group A streptococci at concentrations of the antibiotic just sufficient to inhibit growth. The implication is that members of this group may exert their primary effect upon the protein synthetic machinery. Of these antibiotics, chloramphenicol and the tetracyclines have previously been considered by Gale and Folkes to be specific inhibitors of protein synthesis on the basis of experiments with whole [37] and disrupted cells [38] of S. aureus. The suggestion that chloramphenicol and the tetracyclines affect pathways contributing to protein synthesis was also proposed by Ciak and Hahn [39] on the basis of their additive action on the growth of E. coli. Further evidence has recently been provided by Mager [40] who observed that in a cell-free system from Tetrahymena chloramphenicol and chlortetracycline inhibit amino acid incorporation into protein.

Puromycin hydrochloride and its aminonucleoside were kindly supplied by Drs. E. C. De Renzo and L. Goldman of Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York. The preparation of this article was facilitated by a valuable review of literature relating to the chemistry and biology of puromycin which was prepared by Dr. L. Goldman and made available to us prior to publication.

In consulting the literature the reader should be advised that the generic name Puromycin replaces the older term Achromycin, trade names of Lederle Laboratories Division, American Cyanamid Company. Stylomycin is the current trade name of Lederle Laboratories for its brand of Puromycin, while Achromycin has become the trade name of Lederle's brand of tetracycline.

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DISCUSSION

DR. BROCK: I have several comments based upon my own work and also in relation to this very interesting work. First, with erythromycin we were able to show that it was exactly additive with chloramphenicol and so this would fit in to the same picture with tetracycline that Dr. Hahn mentioned. Secondly erythromycin induced RNA accumulation in the same way as chloramphenicol and apparently the same way that pimaricin does. The RNA accumulated in erythromycin is unstable as is the chloramphenicol induced RNA, provided the instability is sought for in the right way, and I don't want to go into that since we have another paper on this subject which will be in press shortly. Erythromycin induces a lag in the same way as chloramphenicol. In all of our experiments with erythromycin we could not find any differences. Chloramphenicol does not inhibit the activation or transfer to soluble RNA as Dr. Hahn has mentioned, and so it seems to me that we have here two antibiotics, pimaricin and chloramphenicol, which have identical modes of action, at least at this level. It is also very interesting, if I understood this last chart correctly that you had to have an aromatic amino acid on the pimaricin before it was inhibitory. Is that correct? Of course, chloramphenicol is in a sense, an aromatic amino acid or a very closely related one.

Another point. A group in England had a note in the Biochemical Journal three or four months ago that amino acids are incorporated into lipids and that this incorporation is inhibited by chloramphenicol. No details, yet here is another place we must consider in looking for amino acid incorporation. So far as I know, lipids are the stepchildren of biochemists and possibly this is of some importance. It may be a secondary effect since this occurs in the bacterial membrane.

We looked for the inhibition of peptide bond synthesis with both chloramphenicol and erythromycin in an in vitro system of chymotrypsin mediated synthesis of tyrosil tyrosine. However, this synthesis was not inhibited by either of these antibiotics.

The last point is based on Dr. Hahn's work on D-glutamyl polypeptide synthesis, a system which I believe is more important than had been recognized to date, because we have separated growth from protein synthesis. This is a system that seems to me ideal for studying the mechanism of the action of chloramphenicol. Since the L + erythro-isomer is the one which inhibits this system, I would be very interested to know whether any one has tested to see whether this isomer also inhibits cell wall synthesis in bacteria, since in the cell wall synthesis a number of D-amino acids are involved. It seems to me that it is possible that the reason chloramphenicol does not inhibit cell wall synthesis in bacteria, is that the peptide linkages are of the wrong configuration.

DR. C. G. LEONARD (Ft. Detrick): I would like to make some remarks on our work on the effects of chloramphenicol and its isomers on polypeptide synthesis by *Bacillus subtilis* (now classified as *B. licheniformis*), since our findings and conclusions are different from those just presented by Dr. Hahn.

The following background information will be helpful for these remarks. Dr. Hahn's work was done using static conditions for growth of *B. subtilis* in Sauton's medium made in tap water. Under these conditions of growth about 2 mg polypeptide are produced per milliliter culture filtration in about 6 days of incubation. We used shaken conditions for growth of *B. subtilis* in chemically defined Medium E, made in triple distilled water (Leonard et al., J. Bacteriol. 76: 499-505, 1958). Under these conditions of growth about 17 mg polypeptide are produced per milliliter culture filtrate in about 4 days of incubation. In another publication (Thorne and Leonard, J. Biol. Chem. 233: 1109-1112, 1958) we provided evidence that preparations of polypeptide from *B. subtilis* which contain both isomers of glutamic acid, are mixtures of two peptides, one composed predominantly of the D-isomer and the other of the L-isomer. Under our conditions of growth *B. subtilis* doesn't produce any significant amounts of polypeptide with both L- and D-glutamic acid in the same peptide chain. We also reported that increasing concentrations of Mn++ (from $1.5 \cdot 10^{-7}$ M to $6.1 \cdot 10^{-4}$ M) in Medium E (with $1.0 \cdot 10^{-3}$ M CaCl) resulted in increasing production of D-glutamyl polypeptide, between 36 and 80%, without affecting the total yield of polypeptide.

We then studied the effect of chloramphenicol and some of its isomers on polypeptide synthesis using Medium E. We found that the L(+)-threoisomer (50 μg/ml) had no effect on growth or polypeptide synthesis by *B. subtilis*. On the other hand when 50-200 μg DL-erythro isomer were added per ml of culture medium a marked repression in growth was observed for about 20 hr. Polypeptide synthesis was significantly lower than in controls even after full growth was obtained. Increasing concentration of Mn++ ($1.5 \cdot 10^{-7}$ to $1.5 \cdot 10^{-4}$) progressively reversed the inhibition on polypeptide synthesis until no inhibition was evident (4 days of incubation). It was also found that doubling the Ca++ concentration to $2.0 \cdot 10^{-3}$ M and using $1.5 \cdot 10^{-6}$ M Mn++ completely reversed the inhibition produced by 50 μg of DL-erythro isomer per milliliter of medium. In addition, it was found that when inhibition of polypeptide synthesis was observed with DL-erythro isomer both the L- and D-glutamic acid polypeptides were equally inhibited. Also, the inhibition could be related to the slower rate of growth and increased rate of death of *B. subtilis* cells grown in the presence of DL-erythro isomer and low concentrations of Mn++.

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The effect of chloramphenicol [D(-)-threo isomer] on polypeptide synthesis was studied using resting cells of B. subtilis. The percent of D-glutamic acid polypeptide produced by resting cells is determined by the concentration of Mn^{++} used in the initial growth medium. When 10-600 μg of chloroamphenicol were added/milliliter of testing medium there was no inhibition of total polypeptide synthesis, nor did it influence the amount of either L- or D-glutamic acid polypeptide produced.

On the basis of these results, we believe that none of the isomers of chloramphenicol tested has a significant effect on either L- or D-glutamic acid polypeptide synthesis by B. subtilis when both Mn^{++} and Ca^{++} are present in the medium in optimum concentrations for growth and polypeptide synthesis. The inhibition by the DL-erythro isomer on polypeptide synthesis only when trace amounts of Mn^{++} are present might be due to the binding of Mn^{++}.

DR. HAHN: I first would like to answer one question that Dr. Brock has asked. Experiments on E. coli with the four isomers of chloramphenicol have in no case shown spheroplast formation. Growth inhibition does not occur with anything but chloramphenicol itself, so, if you would consider this as circumstantial evidence, it would point to the idea that no overt effect is exerted on the cell wall formation by one of the isomers of chloramphenicol. We have not looked for the accumulation of cell wall precursors and, if this were indeed shown, we would have to reverse our thinking.

I was very much interested to hear about the results which the lady from Ft. Detrick mentioned that the formation of both isomers of polygintamic acid is capable of being inhibited by the same stereoisomer of chloramphenicol. Now, there is one feature about glutamyl polypeptide that I have not mentioned, but I think is important here. Glutamyl polypeptide is a polypeptide with a gamma linkage, that is the gamma-carboxyl of the glutamic acid is linked to the amino group of the adjacent glutamic acid molecules. If we accept her results as conclusive, then it would tend to modify our thinking to assume that there is some relationship between the isomer of chloramphenicol and the nature of the peptide bond that is formed under this condition. Since I heard about this, just as recently as all of you did, I have not been able to think this through in detail, but at least there is one difference where one might look for an explanation. On the other hand, an action of chloramphenicol as a chelating agent, does not seem to have any foundation in the chemical structure of chloramphenicol and from the data of which we are aware, no binding of metals to the chloramphenicol molecule has been shown. I wonder if anyone has a comment on the possible action of chloramphenicol as a chelating agent.

DR. LEONARD: I guess I should have stressed the point that when I was talking about concentrations of Mn^{++}, I was talking of a very small level. For good growth and high levels of peptide, you need $1.5 \cdot 10^{-7}$ M of Mn^{++}, I do know, as he pointed out, first there is a D-isomer which is unusual, second, there is a gamma linkage, which is also unusual. I think the original idea of the mode of action chloramphenicol is not affected by this, but that using this work on polypeptides as additional evidence that, it is certainly stereospecific, I don't think Dr. Hahn's suggestion is valid.

DR. SAZ: I wonder if perhaps there might be some relationship here to the fact that fantastically low concentrations of manganese stimulates spore formations in B. subtilis and this change of state from the vegitative to a spore or a prespore state might have something to do with the situation. Dr. Weinberg at Indiana University has shown that the addition of even $1 \cdot 10^{-8}$ M manganese to a complex medium like nutrient broth will induce the formation of spores, when without the addition of manganese to the extent of $1 \cdot 10^{-8}$ M, you have no spires. It sounds incredible, but this is the situation, and it may have some bearing on this problem. Is there further discussion on these two interesting papers?

DR. STROMINGER: I have several questions for Dr. Hahn and also Dr. Yarmolinsky. For Dr. Hahn, there is one well-defined example of transfer reaction from s-RNA in the synthesis of peptide, i.e., the recently reported example of Bates and Lipman in which a glycine was transferred from s-RNA to form glutathione. I wonder if this system had been every directly tested with chloramphenicol? My second point for Dr. Hahn is that there are many proteins in bacterial cells. Some of them are intercellular, some of them are found in the membrane, and particularly in the Gram-negative bacteria, at least several species of proteins can be found in the cell wall. I wonder if any direct investigation has been made of the effect of chloramphenicol on the synthesis of different proteins in the bacterial cell. I ask this question particularly because of the discussion of the significance of the Belianski and Ochoa paper as to whether this was related to "true protein synthesis" or not. The question is whether cell wall proteins might be synthesized by some mechanism which is different from the synthesis of intercellular proteins and differential effect of chloramphenicol might give some clue to this aspect.

For Dr. Yarmolinsky, I have two questions. One is about the kinetics of the inhibition of transfer from s-RNA to protein. Have any kinetic studies been done, and can you say whether the inhibition is competitive or noncompetitive? I realize that experimentally it may be difficult to do this, but I wondered if they had been tried. Also, I wasn't quite clear about Dr. Nathan's data. The tryosine derivative of primaricin was effective is that right? The second question is: have any derivatives of the amino nucleotide been tested in which the end of the amino acid being transferred was modified? For example, chlorine on the end of leucine or a methoxy group somewhere in the substituted amino acid? Since we have a methyl tyrosine, the question is whether the other end of the amino acid is important in the inhibition?

DR. HAHN: The first question is whether the system of Bates and Lipman that leads to glutothione is susceptible to chloramphenicol. I do not know. The only work on glutothione synthesis and chloramphenicol was presented for the first time in Paris in 1952, and subsequently published in the Biochemical Journal in 1953 from Gale's Laboratory. Here this synthesis was not susceptible to chloramphenicol action. As far as specific proteins and Gram-negative organisms are concerned there is no extensive answer to this. Various enzymes of course are not formed under chloramphenicol action. From the work of one of the other groups at Walter Reed, I remember that the formation of a certain toxin from Pasteurella pestis, was indeed inhibited by chloramphenicol action. As far as other proteins or peptides are concerned, the only work that I am aware of is by Park and his associates published in Nature, Volume 181, in which they showed that the formation of several peptides in Gram-positive organisms was inhibited by chloramphicol.

DR. YARMOLINSKY: This is obviously a very interesting point. As you know, Dr. Nathan has purified an additional enzyme for this process. He intends, I believe, to see whether this additional enzyme might be involved in the action of puromycin. So far, no kinetic data are available. I think this is very likely to be done soon, however. Certainly it's of interest to know if there are any components of the protein synthesizing system which could reverse or alleviate in some way the inhibition by puromycin.

Now, in regard to the second question, concerning the hind end of the amino acid. You might consider that β-alanine, has an active group at the far end of molecule. The β-alanine derivative of puromycin is inactive in the in vitro system. I don't know about any other substituents on the aromatic moiety, aside from the chlorine group. The metachlorine derivative of puromycin is active in the in vitro system.

DR. SAZ: Before we proceed to the next papers, Dr. Perrin Long has just asked a terribly nasty question, and really he has no right to ask biochemists anything of this nature. He points that in the treatment of urinary tract infections, particularly in coliform infections, standard therapy is a mixture of chloramphenicol and streptomycin. The question, therefore, is "What has all of this stuff that you biochemists are presenting to do with the facts of life?" I would like to ask some of these people who have been talking about streptomycin and chloramphenicol antagonisms, etc. to give their very loose comments upon this question.

DR. HAHN: Before even attempting to answer this, may we know the evidence for which this mixture is used in urinary infections?

DR. LONG: If you go around the hospitals of this country today as I have, you will find urinary tracts infections are commonly treated successfully with combinations of chloramphenicol and streptomycin.

DR. SAZ: Do we have any further comments upon this? I think perhaps we had best take the usual refuge of the biochemist, and say that time alone will give the answer to this problem. When we answer in this fashion we generally hope not that time alone will give the answer but that the question will be forgotten.

But, seriously though, I should like to point out that we are not yet far enough advanced in our research on the modes of action of antibiotics to attempt to tie in these biochemical findings with the clinical findings. Certainly, even in general field of enzymology which is much, much further along than our studies on the mode action of antibiotics, except in a relatively few cases, and particularly in certain congenital diseases, we have been unable to make any sort of real honest to goodness correlation which would satisfy both the practitioner and the biochemist. It is a question, of course, which must be before us all the time. Someone has to put these pieces together ultimately.

ANTIBIOTICS AS INHIBITORS OF BACTERIAL CELL WALL SYNTHESIS

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In recent years it has become apparent that several antibiotics owe their selective toxicity for microorganisms to some manner of inhibition of bacterial cell wall synthesis. The bacterial cell wall is a well defined morphological entity, as is also the case in higher plants, and the selective toxicity of some antibiotics is, therefore, due to interference with a structure which does not have a morphological or chemical equivalent in animal cells. Before discussing this phenomenon, it may be useful to define the bacterial cell wall briefly.

Two membranes can be seen in a bacillus stained with Victoria blue [3]. One of these membranes is internal and is very well stained by the dye; it is called the cytoplasmic membrane. The other structure is external, best seen at the ends of cells, and only poorly stained by the dye; that structure is called the cell wall. In encapsulated bacteria, the wall lies between the capsule and the membrane. Both membranes can also be seen in electron micrographs of thin sections of bacteria [4].

A second way of defining the bacterial cell wall came from physiological experiments, first carried out by Weibull [5]. It had long been known that sensitive bacteria, e.g., Bacillus megaterium, can be lysed by an enzyme, lysozyme, which is found in egg white and tears, among other places. However, Weibull observed that if the treatment with lysozyme was carried out, not in ordinary medium, but in hypertonic sucrose, the microorganisms were not lysed. Instead they were converted to spherical forms which were termed protoplasts. The action of lysozyme is to digest the cell wall of sensitive bacteria and these experiments then define the cell wall as the external structure which gives form and shape to the microorganism and serves to protect it from such deleterious influences as osmotic shock. If the spherical protoplasts, with their fragile cytoplasmic membranes, are centrifuged and resuspended in media of ordinary tonicity, they spontaneously lyse.

Two lines of evidence first independently suggested that penicillin might be inhibiting bacterial cell wall synthesis. Duguid in 1946 [6] had observed characteristic morphological alterations in bacteria under the influence of penicillin and recognized their significance. However, his observations escaped attention for over a decade. Later, Liebermeister and Kellenberger [7] observed these changes: first budding, then gradual increase of the size of the bud, and finally the development of spherical forms. This observation was also made by Lederberg [8] who observed the formation of spherical forms of Escherichia coli under the influence of penicillin in hypertonic sucrose broth. When the penicillin was removed, the cells reverted to their normal bacillary forms. Lederberg recognized that these spherical forms were analagous to protoplasts formed under the influence of lysozyme and he therefore inferred that penicillin might be interfering with the synthesis of the cell wall or with its maintenance. A similar observation was made by Hahn and Ciak [9]. This experiment is an extremely simple one and is now carried out in many microbiology courses.

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*This paper is an edited transcript of the paper presented at the symposium. More detailed accounts will be found in references [1] and [2]. The author is greatly indebted to several collaborators, Dr. Eiji Ito, Dr. Stanley Nathenson, Mr. Robert Threnn, and Mrs. Shirly Scott, and to the National Science Foundation (Grant G-7619) and U.S. Public Health Service (Grant E-1902) for support.

The second line of investigation which led to the same hypothesis began with the observation of Park and Johnson [10, 11] that uridine nucleotides accumulate in Staphylococcus aureus inhibited by penicillin. The structure of the principal nucleotide which accumulates under these circumstances is shown in Fig. 1. As

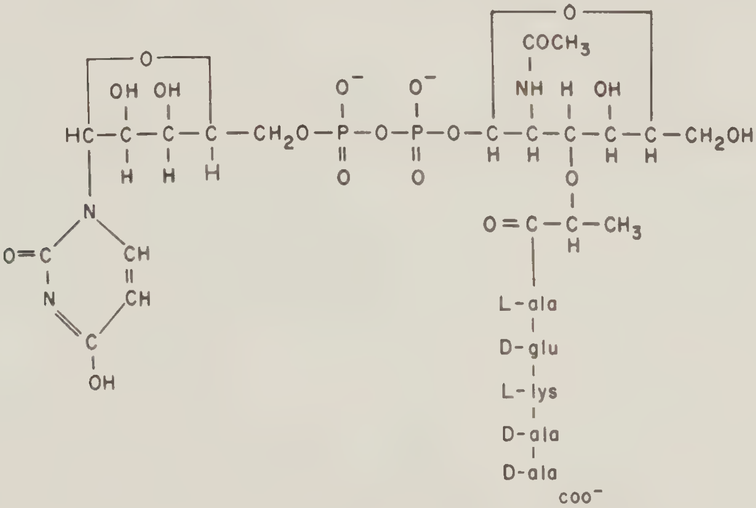

Fig. 1. The structure of UDP-GNAc-lactyl-peptide, a uridine nucleotide which accumulates in penicillin-inhibited S. aureus.

in other compounds of this type uridine is linked through a pyrophosphate bridge to a sugar. The sugar has a most unusual structure. It is a 3-o-lactic acid ether of N-acetylglucosamine. It was first crystallized from a hydrolysate of "spore peptide" by Strange and Dark [12] who named it acetylmuramic acid. The carboxyl group of the lactic acid is substituted by a peptide, the sequence of which is (L)ala-(D)glu-(L)lys-(D)ala-(D)ala [13, 14]. Another unusual feature of the structure of the nucleotide is the occurrence in it of D-amino acids. The discussion will return to this point later, particularly to the fact that (D)ala-(D)ala is at the end of the peptide sequence.

At the time the nucleotides were isolated, little guess could be made as to what sort of function they might have. First of all, data collected showed that the point of attack of penicillin might be very closely related to nucleotide accumulation. Secondly, a great deal of work was done on the qualitative composition of bacterial cell walls which had been prepared by a method developed originally by Salton and Horne [15]. It became apparent from a number of investigations that all bacterial cell walls contain a structure which may be called the basal structure. The invariable constituents of this basal structure are acetylglucosamine, the lactic acid ether of acetylglucosamine and three amino acids, alanine, glutamic acid, and either lysine or α, ϵ-diaminopimelic acid (ϵ-carboxy-lysine). Some bacterial cell walls also contain a phosphate ester which was identified as ribitol phosphate in several cases.

These observations suggested that the basal layer of the cell wall had a structural similarity to the nucleotide which accumulated. A careful quantitative investigation of the structure of the cell walls of several strains of S. aureus which

Table 1. Effects of Penicillin on Incorporation of Isotopes into Cell Wall or into Cell Protein and Nucleic Acid in S. aureus and in E. coli*

Isotope	S. aureus				E. coli	
	C^{14}-lysine		P^{32}-inorganic phosphate		H^{3}-DAP	C^{14}-glucose
	Cell wall	Protein	Cell wall	Nucleic acid	Cell wall	Cell wall
Control	34,800	5,100	155,000	11,600	1,040,000	389,000
+ Penicillin	3,290	4,960	48,900	11,600	297,000	334,000
% Inhibition	91	2	68	0	72	14

*Data are expressed as specific activities, counts/min/mg.

accumulated the uridine nucleotides was, therefore, carried out [16, 17]. These cell walls contained three alanine and one lysine residue per glutamic acid residue and about one residue of acetylmuramic acid, as does the uridine nucleotide. Even more striking was the comparison of the optical rotation of the amino acids in the wall and in nucleotide. In both of these compounds there were two D-alanine residues for each L-alanine; all of the glutamic acid was D-glutamic acid and all of the lysine was L-lysine. These observations, then, suggested that the uridine nucleotide was a bacterial cell wall precursor and that its accumulation was the consequence of inhibition of cell wall synthesis by penicillin.

This hypothesis is also now supported by isotopic measurements of cell wall synthesis. Such measurements have been carried out in a number of laboratories (cf. [18]). For example, with C^{14}-lysine incorporation of this isotope into the cell wall of S. aureus was 91% inhibited under conditions where as incorporation into cell protein was not at all inhibited (Table 1). Similarly, with P^{32}-inorganic phosphate as the wall precursor, incorporation of the isotope into the ribitol phosphate polymer in the cell wall could be essentially completely inhibited under conditions where there was no inhibition of incorporation into nucleic acids. In a Gram-negative microorganism, E. coli, incorporation of tritiated diaminopimelic acid could be essentially completely inhibited by penicillin, although at much higher concentrations of the antibiotic.

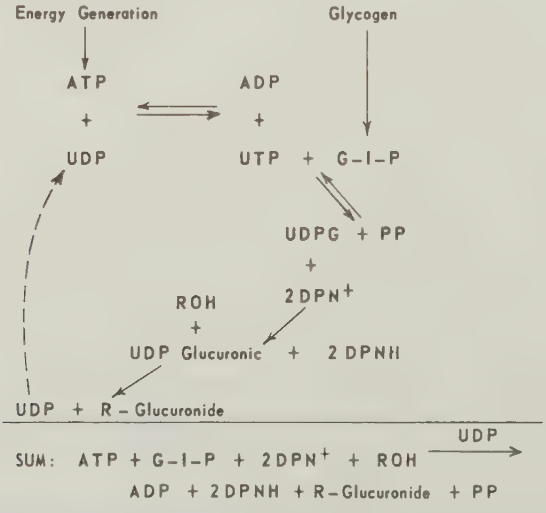

Fig. 2. The over-all reaction in glucuronide synthesis: a UDP cycle, emphasizing the catalytic activity of uridine nucleotides.

It might be profitable to digress briefly at this point to discuss the role of uridine nucleotides in cellular metabolism. A large number of uridine nucleotides are known in which uridine diphosphate is substituted by a sugar fragment, for example, the group of compounds related to UDP-glucose. From UDP-glucose, a number of other sugars can be synthesized. The important feature for the present discussion is that each of these sugars, activated as the nucleotide derivative, can be used in some synthetic reaction, for example, the synthesis of glucuronides from UDP-glucuronic acid, of glycogen from UDP-glucose, or of lactose phosphate from UDP-galactose.

The cycle of glucuronide synthesis will illustrate a typical reaction cycle which leads to the synthetic reaction (Fig. 2). At the beginning of the cycle is the primary phosphorylation reaction, the ATP-dependent conversion of UDP to UTP. The second reaction is the activation of the sugar, that is, the formation of UDP-glucose from UTP and glucose-1-phosphate. The next reactions are modification of the sugar, in this case by oxidation to UDP-glucuronic acid, and finally the transfer of the modified sugar fragment in a synthetic reaction which leads to the synthesis of the glucuronide. The important features of this reaction cycle are phosphorylation, activation of the sugar, and finally its modification and transfer.

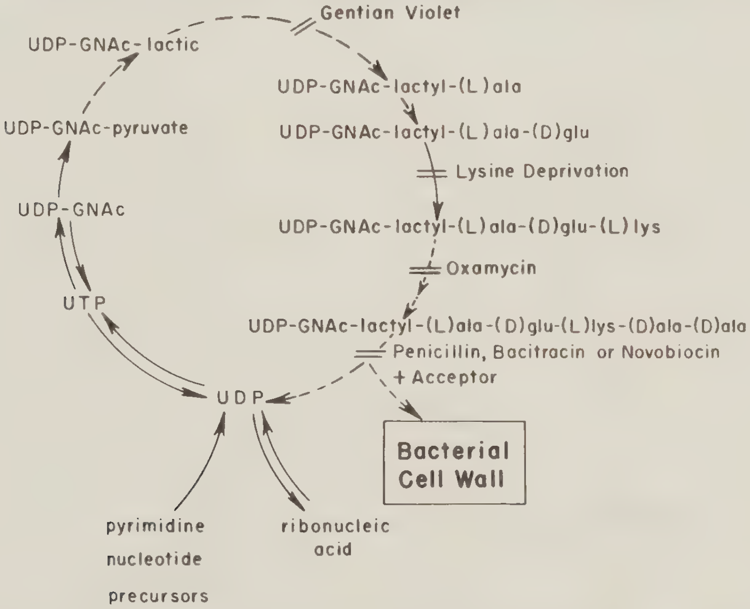
Gentian Violet
UDP-GNAc-lactic
UDP-GNAc-lactyl-(L)ala
UDP-GNAc-pyruvate
UDP-GNAc-lactyl-(L)ala-(D)glu
Lysine Deprivation
UDP-GNAc
UDP-GNAc-lactyl-(L)ala-(D)glu-(L)lys
Oxamycin
UTP
UDP-GNAc-lactyl-(L)ala-(D)glu-(L)lys-(D)ala-(D)ala
Penicillin, Bacitracin or Novobiocin
+ Acceptor
UDP
Bacterial Cell Wall
pyrimidine nucleotide precursors
ribonucleic acid

Fig. 3. The cycle which leads to synthesis of part of cell wall of S. aureus.*

The same kind of reaction cycle takes place in bacterial cell wall synthesis (Fig. 3). At the beginning of the cycle there is also phosphorylation of UDP to UTP and next the activation of acetylglucosamine to form UDP-acetylglucosamine. The sugar is then modified in a complex reaction mechanism in which first a three carbon fragment is introduced at the oxidation level of pyruvate and then reduced to the oxidation level of lactate. Finally, amino acids are added in a stepwise fashion to yield the uridine nucleotide containing the acetylmuramic acid-pentapeptide fragment. This cycle can be inhibited at a number of points. In some manner, not yet precisely defined, penicillin, bacitracin, and novobiocin inhibit at a late stage in the reaction cycle predominantly leading to accumulation of the late intermediate. At earlier stages the cycle may be inhibited by the antibiotic, oxamycin (D-cycloserine), deprivation of L-lysine (an essential amino acid for S. aureus), or an impurity present in some batches of gentian violet. These inhibitions lead to accumulation of earlier intermediates [cf. 1, 2].

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*A similar mechanism (in which meso-diaminopimelic acid replaces L-lysine) operates in synthesis of the basal structure of the cell wall of E. coli. This reaction scheme is similar to the scheme for glucuronide synthesis (Fig. 2). The position of the inhibitors is indicated with reference to the compounds which accumulate with each substance. This representation does not imply that these substances specifically inhibit the indicated reactions.

A similar cyclic mechanism occurs in the synthesis of the basal structure of the cell wall of E. coli. The late intermediate containing meso-diaminopimelic acid instead of L-lysine in the peptide sequence has been isolated from normal cultures of this organism [19]. The E. coli strain used in these experiments was an auxotroph requiring diaminopimelic acid. When it was deprived of diaminopimelic acid, it accumulated the early intermediate containing the "incomplete" peptide, L-alanyl-D-glutamate, which has been shown to be identical to the intermediate which accumulates in S. aureus deprived of lysine. The earlier reactions in the cycle in E. coli are known from enzymatic experiments.

Each of the reactions in the cycle may be described briefly. The activation of the sugar, acetylglucosamine, is best measured in the direction of pyrophosphorolysis of UDP-acetylglucosamine. In the cell the reaction undoubtedly goes mainly in the direction of synthesis of UDP-acetylglucosamine from UTP and acetylglucosamine-1-phosphate [20]. Then, a three-carbon fragment is transferred from phosphoenolpyruvic acid to UDP-acetylglucosamine with the formation of a 3-o-enolether of pyruvate on acetylglucosamine [21]. The next step, the reduction of the pyruvate intermediate to the lactic acid intermediate, has been barely detected. The evidence that the product is the UDP-lactic acid ether derivative (UDP-acetylmuramic acid) is not conclusive. This reaction is the least well studied of the reactions in the cycle.

In the next three reactions [22, 23], three amino acids, L-alanine, D-glutamic acid, and L-lysine, are added sequentially. Each of the separate enzymes which

Table 2. Uridine Nucleotide Requirement in Synthesis of Peptide Bonds*

Uridine nucleotide added	Substrate		
	C^{14}-L-alanine	C^{14}-DL-glutamic acid†	C^{14}-L-lysine
None	0	0	0
UDP-GNAc-lactic	1364	0	0
UDP-GNAc-lactyl-L-alanine		3720	0
UDP-GNAc-lactyl-(L)ala-(D)glu	8	0	3530
UDP-GNAc-lactyl-(L)ala-(D)glu-(L)lys	2200‡	0	20
UDP-GNAc-lactyl-(L)ala-(D)glu-(L)lys-(D)ala-(D)ala	100	0	20

*Data are recorded as counts/min incorporated into the uridine nucleotide precursor. The enzyme preparation was a sonic extract of S. aureus, precipitated with ammonium sulfate and dissolved in buffer.

†Although C^{14} -DL-glutamic acid was the substrate, it could be shown that only C^{14} -D-glutamic acid was enzymatically active.

‡This result is known to be due to the occurrence of alanine racemase and the enzymes catalyzing Reactions 7 and 8 in the preparation.

catalyzes these reactions requires ATP and a divalent cation, in addition to the specific amino acid and a specific uridine nucleotide. Manganese is effective in general at lower concentrations than magnesium. The specificity of amino acid addition for the uridine nucleotide precursor is shown in Table 2.

The last two steps are again catalyzed by separate enzymes but a somewhat different mechanism of peptide bond formation is involved. First, two D-alanine residues are put together in the dipeptide, D-alanyl-D-alanine. The dipeptide is then added as a unit to the uridine nucleotide. During purification of the system in which D-alanine was incorporated into the uridine nucleotide, the ability to incorporate D-alanine was rapidly lost. However, Ikawa and Snell [24] had previously isolated D-alanyl-D-alanine from a strain of *Streptococcus fecalis*. This compound could also be isolated from lysine-deprived *S. aureus* in which situation the dipeptide accumulates together with a uridine nucleotide. Both isolated D-alanyl-D-alanine and a synthetic sample of the dipeptide could be added to the uridine nucleotide precursor (Table 3).

Table 3. Measurement of the Incorporation of Non-radioactive Amino Acids and Peptides into an Enzymatically Synthesized Radioactive Uridine Nucleotide.*

Additions	Counts/min in UDP-GNAc-lactyl-(D)ala-(D)-glu-(L)lys-(D)ala-(D)ala
None	105
(D)ala-(D)ala (synthetic)	4,790
(D)ala-(D)ala (isolated)	6,600
(L)ala-(L)ala	20
(D)ala-(L)ala	56
D-alanine	81
L-alanine	37

*The enzyme was a partially purified preparation.

The reactions which lead to synthesis of the peptide-containing uridine nucleotide are, therefore, the following:

1. UTP + GNAc-1-P $\rightleftharpoons$ UDP-GNAc + PP
2. UDP-GNAc + P-pyruvic acid $\rightarrow$ UDP-GNAc-pyruvic acid ether + Pi
3. UDP-GNAc-pyruvic acid ether $\dashrightarrow$ UDP-GNAc-lactic acid ether
4. UDP-GNAc-lactic + (L)ala $\xrightarrow[\text{Mn}^{+}]{\text{ATP}}$ UDP-GNAc-lactyl-(L)ala
5. UDP-GNAc-lactyl-(L)ala + (D)glu $\xrightarrow[\text{Mn}^{++}]{\text{ATP}}$ UDP-GNAc-lactyl-(L)ala-(D)glu
6. UDP-GNAc-lactyl-(L)ala-(D)glu + (L)lys $\xrightarrow[\text{Mn}^{++}]{\text{ATP}}$ UDP-GNAc-lactyl-(L)ala-(D)glu-(L)lys
7. 2(D)ala $\xrightarrow[\text{Mn}^{++}]{\text{ATP}}$ (D)ala-(D)ala
8. UDP-GNAc-lactyl-(L)ala-(D)glu-(L)lys + (D)ala-(D)ala $\xrightarrow[\text{Mn}^{++}]{\text{ATP}}$ UDP-GNAc-lactyl-(L)ala-(D)glu-(L)lys-(D)ala-(D)ala

Enzymes catalyzing these reactions have been found in nine bacterial species investigated, with some interesting exceptions. The most significant point about the distribution is that those organisms which contain lysine in their cell walls also contain the lysine-adding enzyme (e.g., *S. aureus* and *S. fecalis*). However, those

Table 4. Enzymatic Addition of Either Lysine-C^{14} or of Diaminopimelic Acid-H^3 to UDP-GNAc-lactyl-(L)ala-(D)glu*

Microorganism	Amino acid substrate	
	Lys-C^{14}	DAP-H^3
S. aureus	3000	0
S. fecalis	4770	0
E. coli	0	420
C. xerosis	0	7270

*Data are recorded as counts/min incorporated into the uridine nucleotide and are corrected for a blank from which the uridine nucleotide was omitted. The enzyme preparations were sonic extracts of each organism, precipitated with ammonium sulfate and dissolved in buffer.

organisms which contain diaminopimelic acid in the basal structure of the cell wall do not have a lysine-adding enzyme, but instead they have a diaminopimelic-acid—adding enzyme, e.g., E. coli and Corynebacterium xerosis [24] (Table 4). Therefore, the occurrence of either lysine or diaminopimelic acid in the basal structure of the bacterial cell wall is apparently determined by the specificity of a single enzyme which is able to catalyze the incorporation of either lysine or diaminopimelic acid.

Each of these enzymes (except the one catalyzing reaction 3) has been purified at least 20 times and investigations of the mechanisms of the peptide bond synthesizing reactions are being carried out. In at least three cases (lysine and diaminopimelic acid—adding enzymes and alanyl-alanine synthetase) the mechanisms involve the cleavage of ATP to ADP and inorganic phosphate. They are, therefore, different from the mechanisms of formation of many peptide bonds in proteins which result in the cleavage of ATP to AMP and inorganic pyrophosphate.

Finally, the information just presented has been used to explain in some detail the mechanism of action of one antibiotic, Oxamycin (D-cycloserine). It had been observed by Ciak and Hahn [25] that oxamycin will induce the accumulation of uridine nucleotides in S. aureus. However, it was later shown by two dimensional paper chromatography that one of the compounds which accumulated in the presence of Oxamycin had a slightly different position from one of the compounds which accumulated with penicillin [26]. This compound was isolated in

Table 5. Antagonism by D-Alanine of Uridine Nucleotide Accumulation Induced by Oxamycin

Antagonist added	Experiment 1*	Experiment 2
None	41.4	30.0
D-alanine, 500 μg/ml	17.0	12.1
D-alanine, 5000 μg/ml	4.5	6.9
L-alanine, 5000 μg/ml	41.5	32.2
(DL)alanyl-(DL)alanine, 5000 μg/ml		33.5
D-serine, 5000 μg/ml		34.2

*In Experiment I, Oxamycin (75 μg/ml) and possible antagonists were added together at 0 time. In Experiment 2, Oxamycin (75 μg/ml) was added at 0 time. At 45 min, 20.4 μmoles of nucleotide had accumulated. At this time possible antagonists were added and incubation was continued for 45 min longer. Data are expressed as μmoles of uridine nucleotide per liter of culture at half-maximal growth.

large amounts and subjected to degradation and analysis. It contained only one alanine residue, L-alanine, in addition to one residue each of D-glutamic acid and L-lysine. The structure UDP-GNAc-lactyl-(L)ala-(D)glu-(L)lys could, therefore, be assigned to this nucleotide. The two terminal D-alanine residues from the peptide sequence were missing. This fact, as well as the structure of the antibiotic, suggested that the antibiotic might be a competitive inhibitor of incorporation of D-alanine into the nucleotide. It was readily shown that D-alanine would prevent or reverse uridine nucleotide accumulation induced by the antibiotic in a competitive manner (Table 5) [26]. L-alanine was ineffective in this respect. The only other example of a competitive relationship between substrate and inhibitor in the field of antibacterial chemotherapy is the classical work of Woods and Fildes [27, 28] on the competitive relationship between p-aminobenzoic acid and sulfonamides.

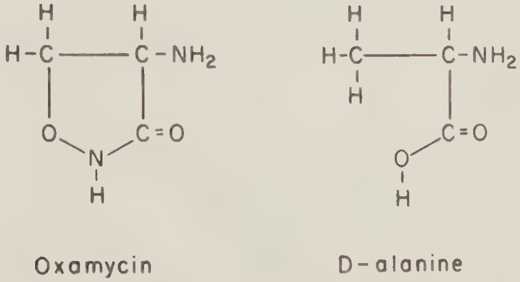
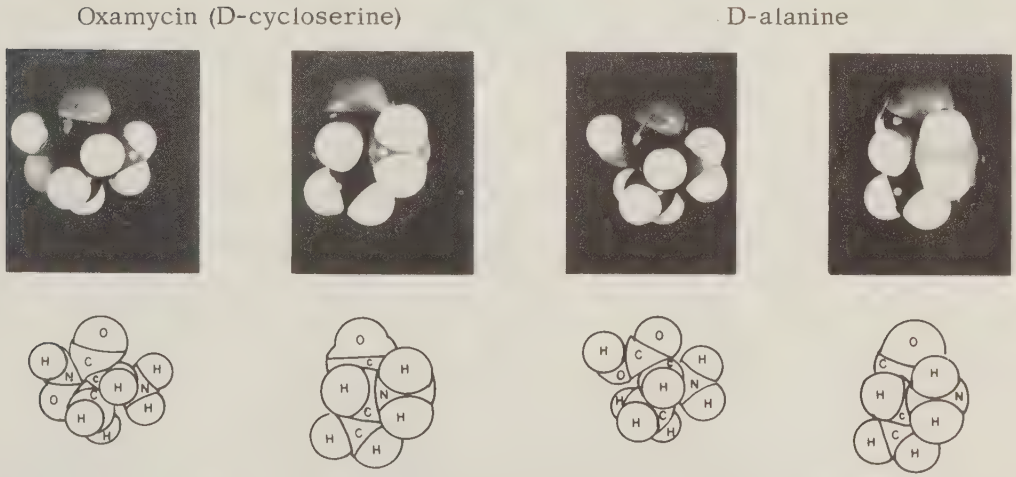


Fig. 4. Structure of D-alanine and Oxamycin.



Oxamycin (D-cycloserine)

D-alanine

Fig. 5. Molecular models of D-alanine and Oxamycin. In each case on the left, the models are viewed looking down on the hydrogen atom on the asymmetric carbon atom. On the right, the models are viewed looking at the amino group on this carbon atom. In the latter view the two structures appear to be identical.

The basis for the competition between Oxamycin and D-alanine is evident from a consideration of the structures of the two compounds. These structures (Fig. 4) are drawn to show the relationship of D-alanine to the antibiotic. Both substances have an asymmetric carbon atom with the D-configuration. The L-isomer of the antibiotic, L-cycloserine, is not a very good antibacterial agent and does not induce uridine nucleotide accumulation. The D-isomer, that is the antibiotic itself, does not prevent incorporation of the L-alanine residue into the peptide sequence. The molecular models of L-alanine structure in comparison with the antibiotic

(Fig. 5) better show the structural similarity which is the basis for the competitive relationship between the antibiotic and the substrate.

Since the sequence of reactions which leads to the synthesis of the peptide was known, the enzymatic basis for the inhibition could also be defined. The two

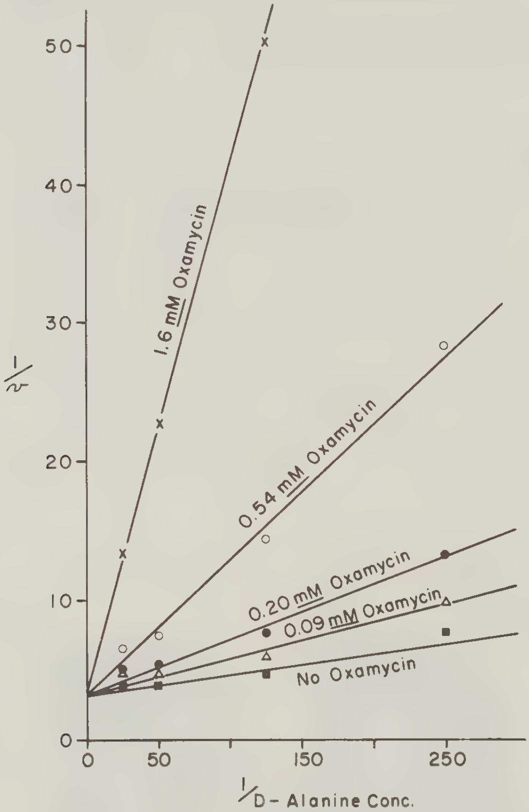


Fig. 6. Competitive inhibition of alanine racemase by Oxamycin. (The kinetic data are plotted by the conventional double reciprocal plot.)

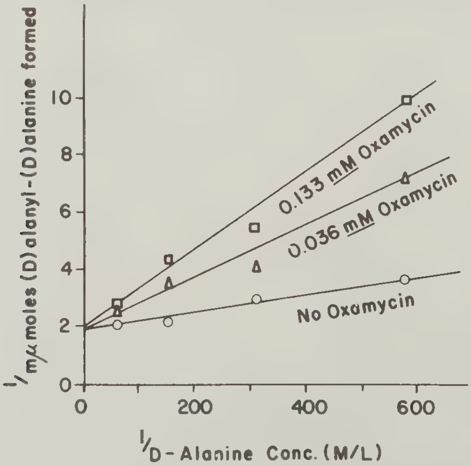


Fig. 7. Competitive inhibition of alanyl-alanine synthetase by Oxamycin.

enzymatic reactions competitively inhibited by the antibiotic are alanine racemase, which catalyzes the formation of D-alanine from L-alanine (Fig. 6); and the enzyme which catalyzes formation of the dipeptide from two molecules of D-alanine (Fig. 7) [29]. The third reaction which is essential for D-alanine incorporation into cell wall, the addition of the dipeptide to the nucleotide, is not at all inhibited by the antibiotic.

From the data shown, the Michaelis constants for D-alanine, that is, the reciprocal of the affinity of the substrate for the enzymes, can be calculated as well as the inhibitor constants of the antibiotic (Table 6). In each case the inhibitor constant is 100 times smaller than the Michaelis constant. That is, the inhibitor (the antibiotic) is bound by the enzyme 100 times as effectively as its natural substrate. In this fact lies the effectiveness of oxamycin as an antibiotic.

Table 6. Kinetic Constants

Enzyme	D-alanine, K_m	Oxamycin, K_i
Alanine racemase	$6 \cdot 10^{-3}$ M	$6 \cdot 10^{-5}$ M
Alanyl-alanine synthetase	$4 \cdot 10^{-3}$ M	$3 \cdot 10^{-5}$ M

There are many directions in which this kind of research can now go. To mention only a few, since the mechanism of action of this antibiotic is now known, it may be possible to determine how bacterial cells become resistant to it. Furthermore, since the molecular basis for its activity is apparent, it may be possible to modify the structure in an intelligent way so as to obtain an even more effective antibacterial substance. Continued search may uncover other antibacterial substances which also owe their selective toxicity to inhibition of bacterial cell wall synthesis.

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INHIBITION OF SYNTHESIS OF BACTERIAL MUCOPEPTIDE OR PROTEIN BY CERTAIN ANTIBIOTICS AND ITS POSSIBLE SIGNIFICANCE FOR MICROBIOLOGY AND MEDICINE

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The discussions this morning have served to emphasize that a number of the useful antibiotics owe their action to interference with essential syntheses of the bacterial cell. Furthermore, the antibiotics most commonly used in therapy are the best understood in terms of mode of action and surprisingly, either protein synthesis or cell wall mucopeptide synthesis is the site of action.

I propose to consider the significance of this conspicuous finding, but before doing so it might be well to take note of other groups of antibiotics which have perhaps received less attention. The polypeptide antibiotics (gramicidin, polymyxin, subtilin, tyrocidin, etc.) are believed to act by virtue of their ability to combine with and disrupt lipoprotein membranes [1]. However, the macrolids (erythromycin, magnamycin, picromycin, spiramycin, etc.), which contain a large lactone ring, an unusual dimethylamino sugar, and sometimes an additional unique sugar, are an important group of antibiotics whose mode of action remains unexplained. The vast majority of antibiotics, it should be noted, are toxic to animal cells as well as microbial cells and again, the basis for the activity of these compounds is largely unknown. Nevertheless, the study of these toxic antibiotics should contribute greatly to our knowledge of living cells. It is to be hoped that interest in this area will increase especially since the pioneer survey by Lardy, Johnson, and McMurray [2] has already indicated that a number of toxic antibiotics interfere with respiratory and phosphorylating systems in mitochondria and should be valuable tools for studying these pathways. Undoubtedly, antibiotics can be found which inhibit other essential metabolic sequences and hence would be of value in elucidating these pathways.

The property of antibiotics which makes them unique for detecting pathways is that those effective in low concentration inactivate a specific enzyme protein by irreversibly combining with it. To determine the site of the block of any macromolecular synthesis, now a relatively simple matter, a radioactive precursor of the polymer is added to the cells under conditions for synthesis and its rate of incorporation into the macromolecule is taken as a measure of synthesis. By comparison of the rate of incorporation into whole bacteria with and without antibiotic one can determine which syntheses if any, are inhibited, provided a satisfactory method for fractionation of the bacterial cells is available. One such fractionation scheme which has proven useful with Staphylococcus aureus as the test organism is given in Table 1 [3]. The key step in this procedure is treatment with hot trichloroacetic acid which hydrolyzes nucleic acids and teichoic acids and renders the protein susceptible to enzymatic digestion. Upon subsequent treatment with trypsin one obtains a soluble fraction which contains about 95% of the protein and a residue which contains the mucopeptide of the bacterial cell wall.

Table 2 summarizes some results obtained with various antibiotics by application of this fractionation procedure [4, 5]. Similar results have been obtained

Table 1. Outline of Fractionation Scheme for Staphylococcus aureus (From Park and Hancock, 1960)

Fraction	Contents of fraction
Cold trichloroacetic acid	Low molecular weight compounds
Alcohol-soluble	Alcohol-soluble "proteins" and lipids
Hot trichloroacetic acid	Breakdown products of nucleic acid and teichoic acid
Trypsin-solubilized	Trypsin-degraded proteins
Residue	Mucopeptide

Table 2. Correlation of Inhibition of Mucopeptide Synthesis With Accumulation of Precursors (UDPX)

Inhibitor, μg/ml	UDPX formed μmole/g	Lysine incorporation	
		Mucopeptide μmole/g	Protein μmole/g
None	5	25.0	3.2
Penicillin, 5	40	5.7	3.7
Oxamycin, 100	29	9.0	2.9
Bacitracin, 50 (2.4 units)	59	1.5	3.4
Glycine, 0.6 molar	21	11.0	3.1
Chlortetracycline, 50	26	4.5	0.2
Chlortetracycline, 10	5	25.0	0.5
Tetracycline, 50	2	24.0	0.4
Chloramphenicol, 50	4	24.0	3.2

by Mandelstam and Rogers [6]. As can be seen from the data in the column entitled "Mucopeptide," penicillin, bacitracin, oxamycin, 50 μg/ml of chlortetracycline, and even glycine at a concentration of 0.6 M inhibits cell-wall mucopeptide synthesis as measured by the incorporation of lysine into this fraction. However, in the same cells in the same experiment, lysine incorporation into protein was not inhibited except by chlortetracycline which is an effective inhibitor of protein synthesis. At a lower concentration, this antibiotic inhibits protein synthesis without affecting mucopeptide synthesis so that it can have two separate actions. Notice that, even at the high concentration, the closely related compound, tetracycline, inhibits only protein synthesis and does not affect incorporation into cell-wall mucopeptide. I was particularly interested in this result because we had known from earlier work [7] that this concentration of chlortetracycline, but not of tetracycline, caused accumulation of the uridine-nucleotide-linked precursors of mucopeptide. The reason for this difference in the tetracyclines has not been investigated further. All the conditions which inhibited incorporation of amino acids into the cell-wall mucopeptide caused accumulation of the uridine precursors and those antibiotics which did not inhibit mucopeptide synthesis did not cause the precursors to accumulate. Thus, these data show a very clear and direct relationship between the accumulation phenomenon and inhibition of mucopeptide synthesis, and demonstrate the independence of the synthetic pathways for mucopeptide and protein. (It may interest those of you tempted to utilize this technique for classification of new antibiotics to learn that all the data in this slide were obtained in one experiment, in one day, by one man. It is so simple, our medical students perform an abbreviated version of it in a three-hour laboratory period.)

We have seen, from this and from the work presented by other members of the panel, that some important antibiotics, such as penicillin, inhibit mucopeptide synthesis, and that other important antibiotics, the tetracyclines and chloramphenicol, inhibit protein synthesis. It seems appropriate that this gathering consider certain implications to be derived from these facts. It is my feeling that our current knowledge of the action of antibiotics, which has contributed to our new insight into the structure and growth of bacteria, has very great significance for the science of microbiology. In fact, general truths have become apparent from this work and from genetic studies which will surely alter the systematization of the present descriptive knowledge that is the basis of this science.

The past four years have witnessed an unusually rapid acceptance of the concept that the bacterial cell is essentially a protoplast protected and preserved by the mucopeptide present in the cell wall.

The fundamental similarities of microorganisms are coming to be recognized as the similarities of their mucopeptides and the rather general susceptibility of bacteria to lysozyme. The very wide antibacterial spectra of the antibiotics, including penicillin, have been revealed. These similarities and the new understanding of the structure of bacteria has given the subject a firm base for investigating relationships among microorganisms, for furthering the development of new antimicrobial drugs, and for predicting the meaning of various observations related to the growth of bacteria. For instance, one can now say with some certainty that any factor which alters the shape of bacteria is doing so by a primary effect on the synthesis or breakdown of cell-wall mucopeptide [8]. As an example of the validity and strength of the present concept, I would like to call your attention to two predictions made in a paper written in the spring of 1957. At that time it was known that Lactobacillus bifidus variety Pennsylvanicus required β-linked N-acetylglucosamine for growth ("bifidus factor" [9]) and that without this abnormal forms and spheroplasts were produced. Assuming that a mucopeptide wall preserves the shape and integrity of bacterial cells, it was predicted that the bound N-acetylglucosamine was the source of amino sugar for synthesis of wall. Confirmation of this prediction has come from Zilliken, who has shown that the muramic acid of the L. bifidus wall is formed directly from the added "bifidus factor" [10]. Presumably the N-acetylglucosamine of the mucopeptide of this organism also originates from added "bifidus factor" and this mutant cannot synthesize UDP-N-acetylglucosamine except from external β-linked N-acetylglucosamine. Another prediction made at that time was that oxamycin acted as an inhibitor because it was an analogue of D-alanine and owed its antibacterial activity to interference with the incorporation of D-alanine into the UDP-N-acetylmuramylpeptide which was believed a necessary precursor of mucopeptide. The basis for this prediction was the observation of Ciak and Hahn [11] that oxamycin caused accumulation of bound N-acetylhexosamine in S. aureus H and the prediction has now been borne out by the work of Strominger, Threnn, and Scott. [12]

Now that I have convinced you that (in the strict sense of the word) bacteriology is becoming scientific, I would like to make three observations which I think should be of value to this group. First of all, consider the observations of Jawetz [13] that frequently synergism occurs in a group of antibiotics consisting of penicillin, streptomycin, bacitracin, neomycin, and polymyxin. Could this mean that two antibiotics acting at different points on the same synthetic pathway are involved to product synergism? Since we believe that penicillin and bacitracin block mucopeptide synthesis, a possible explanation of this synergism with streptomycin, neomycin, or polymyxin would be that these compounds all interfere with protoplast membrane function, some indirectly by interference with mucopeptide synthesis, others more directly, but the combined result is a more rapid destruction of membrane function. The observation that synergism virtually never occurred among members of another group consisting of tetracyclines, chloramphenicol, erythromycin, and novobiocin could indicate that the several inhibitors of protein synthesis act at the same step. Another observation by Jawetz is that a member of group I which is rapidly bactericidal may be antagonized by a member of group II. This would be expected with a penicillin-like antibiotic, which depends on unbalanced growth of the cell for bactericidal action, if that growth were slowed by an inhibitor of protein synthesis. Thus, the results of Jawetz's experiments are consistent with present theory and the theory indicates where further experimental work should prove fruitful.

My second observation is that a fairly close biochemical relationship of the streptomyces to bacteria has become apparent, and I wonder if this fact has had any impact on the screening programs for new antibiotics. For perfectly correct reasons, the emphasis in this search has been on the streptomyces. However, since streptomyces cells possess a small but essential mucopeptide component in their wall, and many are sensitive to lysozyme and to penicillin, we can see that they are very similar to bacteria. This being the case, and since the chances of finding a new antibiotic from a Streptomycete have decreased considerably, it would seem well to consider again for screening purposes those related organisms, that is, the bacteria themselves, such as the aerobic spore-forming bacilli and other important groups found in soil.

Thirdly, I suggest that one must ask why it is that so many of the useful antibiotics act by interference with mucopeptide or protein synthesis. Are these the only essential areas in which animal and bacterial cells differ sufficiently so that a selective toxicity can be expressed Certainly one must be driven to the conclusion that the opportunities for selective toxicity are much more limited than one had every right to expect before the present facts were available. For this reason it seems to me that our existing knowledge of the basis for selective toxicity should be vigorously applied in the search for useful chemotherapeutic agents.

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DISCUSSION

GEORGE GALE: I have two questions for Dr. Strominger. One. Is the alanine racemase in your system pyridoxal phosphate dependent? If it is, have you ever tried to reverse the activity of oxamycin with pyridoxal phosphate? I say this because cycloserine is most active against Mycobacterium tuberculosis, along with isoniazid, which apparently is a pyridoxal inhibitor. The second question is, you showed that oxamycin apparently inhibited both the racemase reaction and the formation of the dipeptide. You also said, I believe, that cycloserine was sterospecific in that the D-form was active, but the L-form was not. Now, in the first of these reactions a substrate is L-alanine, and in the second reaction the substrate is only D-alanine. Is there any apparent conflict here in terms of the activity of D- and L-cycloserine?

DR. STROMINGER: The first question about pyridoxal phosphate: the alanine racemase is dependent on pyridoxal phosphate but it is not competitive with cycloserine over a very wide range of concentration. The second question: The competitive relationship between oxamycin, L-alanine and D-alanine, with alanine racemase is very interesting. The effect of the antibiotic on that particular enzyme is competitively reversed both by L-alanine and by D-alanine, depending on the direction of measurement. There is some evidence that the inhibitor constants (K·) in the two directions are not identical, which may be an artifact. Further study may lead to some interesting information about the mechanism of action of that particular enzyme. We have just obtained some L-cycloserine and plan to test it very shortly as an inhibitor of alanine racemase.

DR. NEWINGBERG: I have a question for Dr. Strominger. Will RNAase inhibit the corporation of amino acids into the nucleotide and if not, what sort of mechanism do you inhibit?

DR. STROMINGER: At first, we believed that the peptide synthesis was RNA-independent. After Bates and Lipmann published a note on the role of RNA in glutathione synthesis, we investigated again. Four of the five reactions are completely insensitive to RNAase. The fifth, alanyl-alanine synthetase, is probably insensitive but there is still some question about it. The mechanism of that reaction in Staphylococcus aureus appears to be extremely complex. It looks like a very simple reaction, but it requires a heat-stable cofactor which has not been identified. There is also some very peculiar activation effect, and a very slight RNAase effect which, I think will turn out not to be related to RNAase. However, I am not willing now to say for certain that the reaction does not depend on RNA although the heat stable cofactor does not seem to be RNA.

DR. PARK: While we are on the subject of the amino acid activation sequence in addition to the nucleotide, I would like to ask if you have any opinion of what is activated and what is the acceptor in the reaction. It looks as though amino acids are being put on from the wrong end. And, if I might ask my other question now, you show that oxamycin is bound 100 times more effectively to the enzyme than the natural substrate. This is much better, I believe, than any organic chemist has been able to do in designing an inhibitor as an analogue. Why do you think that the chemist can improve on it? I would also like to know why the high concentration is required to inhibit microorganisms, since it is such a good inhibitor? Do you know the D-amino acids levels inside the bacteria? They must be almost infinitely large.

DR. STROMINGER: The first question was about peptide or amino acid activation. At first, isotope exchange studies suggested that the carboxyl group of the peptide on the nucleotide was the activated group. However, recently we have been unable to repeat these experiments, and we are, therefore, uncertain as to which group is activated. In any case, the free amino acid does not catalyze any exchange reactions.

Extracellular concentrations of oxamycin required to inhibit bacteria are of the order of 10^{-4}–10^{-3}molar both in vitro and in individuals undergoing therapy. Unfortunately, the intracellular concentrations of the antibiotic and of D- and L-alanine are unknown. There may be a reasonably high concentration of D-alanine inside bacteria, as is known for several other amino acids.

Another question, how chemists could benefit from what we know about the molecular basis for the competition, gives me an opportunity to say something that I didn't have a chance to say before. D-alanine can exist in a number of configurations. There are several centers of rotation in the molecule and in photographing the structure, we chose the one that looked most like the antibiotic. This shape must be the one the substrate has in its reaction with the enzyme. The fact that it can rotate while the antibiotic cannot may be the basis for the higher affinity of the antibiotic for the enzyme. That is, when D-alanine fits into the keyhole on the enzyme it can rotate and wiggle out. The antibiotic has very restricted possibilities of rotation and it may not be able to wiggle out in the same way that the substrate can. What this tells the organic chemists is that there is a shape of the substrate which is important. This shape is given by the oxamycin molecule. Modification of the oxamycin structure must take into account preservation of the face of the molecule which is important in fitting into the keyhole. That's all we can say now.

Q: Can I ask Dr. Strominger if he has any information about the effect of the L-isomer of cycloserine on enzyme activity? Secondly, does he have some information on the relationship between protein synthesis in bacteria cells and the activity of D-cycloserine?

DR. STROMINGER: I don't know the answer to either of those questions. We have just gotten the L-isomer but we haven't tested it. As to the second question, we have not measured any effect on protein synthesis.

DR. BROCK: I would like to make one comment about selective toxicity which has been mentioned here. In a very recent paper in the Journal of Experimental Medicine, Szbalski's group reported some experiments on the effect of chloramphenicol on the growth of human cells, and actually, surprisingly enough, chloramphenicol is highly active in the human cells. It inhibits growth at about 20 μg/ml, which is a concentration we use to inhibit most bacteria. Consequently, I think we have to consider, when using antibiotics in therapy, that we do not only have a host and a parasite but we have a host-parasite relationship and this must be taken into consideration when we discuss how these antibiotics actually work. Bacteria in an infected animal grow at a rather rapid rate. Perhaps chloramphenicol is able to inhibit this growth enough for the nonspecific mechanisms of the host to take over and remove the parasite. Of course, the removal of the parasite is irreversible. Or it is possible, since animal cells may be slower-growing, for one reason or another, that they are not affected as much, so that an antibiotic like chloramphenicol can be very effective clinically even though it might be toxic to the growth of the host cell. It seems to me this may be related to the blood dyscrasia situation that arose a number of years ago. Blood cells are formed during a relatively rapid growth process and if sufficiently high concentrations of chloramphenicol were getting to the place where these cells are growing, this would be effective in deranging metabolism. So, we can't assume that this antibiotic is as highly selective in some molecular way as it would appear, unless we really understand the host-parasite relationship.

DR. HAHN: First of all I fully agree that there are at least two types of selective toxicities possible. One is based on differences in some biochemical qualities and the other is based on rate differences. There is a considerable body of information which indicates that high concentrations of chloramphenicol, up to 1,000 μg/ml or higher, were required to affect the growth of mammalian cell cultures. Hopps and coworkers at the National Institutes of Health had indeed succeeded in curing rickettsial infected cell cultures of the infection by prolonged exposure to chloramphenicol. There was a clear difference after a considerable length of treatment, I believe it was a few weeks. These cells were free of Rickettsia infection. If exposure was carried on farther for three months more some degenerative effects presumably referable to chloramphenicol action occurred. I assume that the work by Szybalski referred to must involve some rather special case which is in contrast to the usual chloramphenicol action on cells in culture.

DR. PARK: On this same subject, I don't think it is really necessary to conclude that protein synthesis in bacteria goes by a different pathway from that in animal cells just because the animal cells apparently are not affected by chloramphenicol. My reason for saying this is that bacterial populations are very diverse so that we have, with regard to cell wall inhibition by penicillin, for instance, a factor of one hundred thousand or even a million, between the most sensitive organisms and the least sensitive. In all of the cells penicillin apparently inhibits the same reaction. So, it may be just a happy circumstance that animal cells, which are a more uniform population, are outside the upper range of sensitivity to the antibiotics.

DR. STROMINGER: I just want to make one other comment on a possible basis of selective toxicity, namely, penetration. As all of you know, bacteria have very prominent mechanisms for concentration of substances from the medium, and they may be different from permeases found in animal cells. This point was brought home when we studied the penetration of oxamycin. We thought that oxamycin might compete for penetration of D-alanine into the cells. In fact, both D-alanine and oxamycin are actively concentrated. However, the permeases are distinct and D-alanine does not compete with oxamycin for penetration or vice versa. I think penetration, in terms of selective toxicity, is something that we have to consider.

DR. SAZ: I might suggest that one topic which has not been covered at all, or just touched on lightly in this discussion this morning, has been the biochemical differences which may exist between sensitive and resistant cells to these various antibiotics, and I put forth the suggestion to whomever will manage the symposium next year that this might very well be a topic for discussion.

THE TREATMENT OF ACUTE GONOCOCCAL URETHRITIS WITH POTASSIUM α-PHENOXYETHYLPENICILLIN*

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INTRODUCTION

In recent years, reports have emanated from a number of countries concerning the increased failure rate of repository penicillin therapy in gonorrhea. Although the penicillin sensitivity of the gonococcus in patients before treatment has usually ranged from 0.002 to 0.333 unit/ml, there is little definitive evidence that this organism generally has developed either increasing or absolute resistance to penicillin. Most of the failures have occurred in patients whose gonococci were less sensitive to penicillin. Gonococci from so-called penicillin-resistant cases usually have been found to be susceptible to penicillin and clinical failures must be attributed to inadequate penicillemia and other factors.

In a comprehensive article on this subject, Willcox [1], in Great Britain, noted that there had been a particular increase of therapeutic failures with repository penicillins in West Indian immigrants, that the infection rate among them had increased about five times during a period of five years, and that the failure rate with available repository penicillins, phenoxymethylpenicillin by mouth, or streptomycin from 1956 to 1957 was 25.2% in a group composed chiefly of West Indians, as compared to 12% in a group composed mainly of native Britons. He considered the probability of a high rate of reinfection in the former group, and also the possibility that less-sensitive strains were being encouraged to develop more quickly as a result of frequent treatment with penicillin of the many repeated infections that occur.

It should be pointed out that reinfection as well as new infections in such a segregated group of persons reasonably could be caused by a more rapid transfer of "naturally" less-sensitive strains in a circumscribed ecology.

Barile et al. [2] reported that seven of 165 cases of gonorrhea seen during 1957 failed to react satisfactorily to three intramuscular doses of 600,000 units each of procaine penicillin G administered daily. Retreatment of three of these patients with five daily doses of 600,000 units of procaine penicillin G had no influence in two. The remaining four patients who were not retreated with penicillin were "cured" with total dosages of 5 g of chloramphenicol administered orally for a period of five days.

In these seven cases of penicillin failure the tolerance of Neisseria gonorrhoeae reached 0.125 unit/ml in three, 0.25 unit/ml in three, and 0.55 unit/ml in one. The tolerance of one susceptible control strain, cultured from a patient who had responded to penicillin therapy, reached 0.015 unit/ml.

All of these instances of penicillin failure occurred in individuals who previously had taken prophylactic doses of oral penicillin G tablets, and who were exposed to contacts in a venereal pool commonly accustomed to taking penicillin G tablets prophylactically.

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*The trade name of J. B. Roerig and Co., Division of Chas. Pfizer & Co., Inc., for potassium phenoxyethylpenicillin is Maxipen.

It might be supposed in these cases that the levels of penicillin attained in the blood and tissues were insufficient to eradicate resistant strains induced by at least two factors, exposure to prophylactic dosage of penicillin and exposure to a venereal pool from which highly susceptible strains had been removed.

As a result of a study based on clinical symptomatic observations, Thurman [3] reported that 11% of 22 patients who had received initial 500 mg doses of potassium phenoxymethylpenicillin (penicillin V potassium) followed by four 250-mg doses every 6 hr had no apparent relief of acute gonorrhea. He contrasted these results with only 3% of failures obtained in the same clinic when single intramuscular injections of 1.2 million units of procaine penicillin in oil with 2% aluminum monostearate had been given.

In a comparative antibiotic study, reported two years ago [4], using recommended "standard" dosages of 1.8 million units of potassium phenoxymethylpenicillin administered orally in three doses of 600,000 units each at 6-hr intervals, we found 16% failures. Subsequently, by increasing the total dosage to 2.4 million units administered in two doses of 1.6 million units initially and 800,000 units 12 hr later, the failure rate was reduced to less than 8%. It should be noted that in both series the possibility of reinfection was considered.

Using the latter dosage as a basis, the presently reported studies were commenced to determine the clinical effectiveness of potassium α-phenoxyethylpenicillin in the treatment of acute gonococcal urethritis in the male.

Alpha-phenoxyethylpenicillin is one of the newer synthetic penicillins. It has been found to be remarkably stable in acid solutions and to be absorbed effectively from the gastrointestinal tract. After this point, there is considerable divergence of opinion regarding the degree of penicillemia obtained after equivalent doses of potassium phenoxymethylpenicillin and α-phenoxyethylpenicillin. As reported by Griffith [5] using serial tube-dilutions and a pathogen as the indicator organism, potassium phenoxymethylpenicillin produced earlier and greater antibiotic activity in the sera than α-phenoxyethylpenicillin. At 2 hr following administration, however, there was considerably less difference in antibacterial activity of the sera produced by the two penicillins. It should be noted that Griffith did not report comparative levels obtained with these two penicillins and that the antibacterial activity was determined with but two different indicator pathogens.

Garrod [6], comparing penicillins V and G with α-phenoxyethylpenicillin, reported that in the range of moderate resistance 12 of 19 strains of staphylococci were more susceptible to synthetic penicillin than to penicillin V, five were equally susceptible to penicillin V and synthetic penicillin, and two were less susceptible to the synthetic penicillin. Other observers have reported that peak levels were obtained at 1 hr after dosage with the synthetic penicillin, and that these peak levels were twice the value of those obtained with phenoxymethylpenicillin.

MATERIALS AND METHODS

One-hundred consecutive male West Indian patients with acute gonorrhea, ranging in age from 18 to 39 years, were treated with potassium α-phenoxyethylpenicillin. The dosage of this synthetic penicillin was based, as indicated above, on our experience with potassium phenoxymethylpenicillin in the treatment of acute gonococcal urethritis. Thus, initially, 1.6 million units (1000 mg) were administered orally followed in 12 hr by 800,000 units (500 mg).

The diagnosis of gonorrhea was determined by a history of exposure, local symptomatology, and physical evidence as well as the finding of gonococci in Gram-stained spreads of urethral exudate. Only those patients with acute gonorrhea were considered for evaluating the efficacy of this dosage of potassium α-phenoxyethylpenicillin.

The patients, after receiving this dosage of potassium α-phenoxyethylpenicillin, were told to return for reexamination on the second or third day, the seventh or eighth day, and again at two weeks posttreatment. At these times spreads and cultures of urethral exudate or discharge, if any, were made and examined for gonococci. When bacteriological examinations were negative at the first two posttreatment visits and the symptoms and clinical manifestations had disappeared, cure was thought to have been reasonably accomplished.

THERAPEUTIC EFFECTS

Using these therapeutic criteria, 96 of the 100 patients who were treated with this dosage of potassium α-phenoxyethylpenicillin reacted satisfactorily and were considered to have been cured. The other four patients, although three were clinically and bacteriologically negative at the first two posttreatment examinations and one was negative at the first posttreatment examination, again presented clinical and bacteriological evidence of gonococcal urethritis at two weeks and eight days posttreatment, respectively. Three of them admitted to having been exposed to the same infectious contacts from whom they had acquired their previous infection. Although these cases might be thought to have relapsed and accordingly considered as penicillin failures, it is quite possible that at least three of the four were reinfected. Actually, when all four patients were retreated with the same dosage of potassium α-phenoxyethylpenicillin they responded in a uniformly satisfactory manner.

In all of these cases the symptoms of urethritis were ameliorated within 12 hr and within a day the urethral discharge had stopped.

TOXIC REACTIONS

There were no notable adverse reactions with this dosage of potassium α-phenoxyethylpenicillin. It is recognized, however, that serious reactions might not be encountered in cases treated with this relatively small short-term dosage. There were no instances of allergic or anaphylactic reactions in any of these patients.

SUMMARY AND DISCUSSION

The cure of 96 of 100 patients with acute gonococcal urethritis attained with a total dose of 2.4 million units (1500 mg) of potassium α-phenoxyethylpenicillin would seem to indicate that for this West Indian population this dosage currently is close to optimal. This contention is supported by an indication that at least three of the four patients in whom cure was thought not to have been obtained with the initial dosage were probably instances of reinfection.

It would seem that potassium α-phenoxyethylpenicillin (Maxipen) is somewhat more effective than potassium phenoxymethylpenicillin in the treatment of acute gonococcal urethritis in males.

Also, despite the increased failure rates of penicillin-treated gonorrhea in some other areas of the world, notably among West Indians in Great Britain exposed to a circumscribed venereal pool that may have a high incidence of infection with less susceptible gonococci, we, as yet, have no clinical evidence that penicillin resistant strains have developed in our own noncircumscribed West Indian ecology.

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ORAL PENICILLIN (POTASSIUM PHENETHICILLIN)* IN THE TREATMENT OF GONORRHEA

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INTRODUCTION

No clinical paper dealing with the treatment of venereal disease should get started without at least mentioning, first of all, the resurgence of V.D. as a grave national health problem and, secondly, the pressing responsibility of all physicians to take, whenever an opportunity presents itself, full educational and medical measures to control the vicious infectious process. Without recourse to statistics the authors believe that the incidence of social disease in this country has been and is rising alarmingly; medical complacency about the problem is simply not warranted by known circumstances. Even though many agencies of various American institutions—social, religious, educational, governmental, and legal—bear an equal responsibility with medicine for educating the public in the control of venereal diseases, the ultimate responsibility for treatment must necessarily fall on the entire medical profession, and not only that part of the medical fraternity engaged in public health programs.

The purpose of this paper is to report a clinical trial of a new oral penicillin in the treatment of gonorrhea. One of the pharmaceutical advances within the last year was the widespread commercial availability of oral potassium phenethicillin. In the treatment of gonorrhea, as in the treatment of many other infectious diseases, penicillin has continued to be remarkably effective throughout the years and is generally accepted as the antibiotic of choice.

Nonetheless, the conventional penicillins have presented the clinician with some disturbing problems which might be summarized, according to Douthwaite [1], as the necessity for repeated injections of penicillin to control acute infection, the remarkable increase in the size of the dose needed to be effective, the pain pro- duced by these injections, the failure of oral penicillin V to control acute infection, the alarming increase of resistance of many organisms to penicillin, e.g., staphylococci and increasing incidence of allergic reactions to intramuscular penicillins. Clearly, one target of research in this field has been the replacement of injectable penicillins with an equally effective oral penicillin.

Oral potassium phenethicillin is a combination of α-phenoxyethyl radical with 6-aminopenicillanic acid nucleus. This has resulted in a penicillin stable in stomach acid. Blood and urine concentrations of potassium phenethicillin indicate that absorption from the alimentary tract is much greater than with penicillin G and twice that of penicillin V in the first hour [2]. The serum protein affinity of potassium phenethicillin is approximately equal to that of penicillin V [3]. The higher penicillin peak concentration in the blood stream with this drug provides longer duration of inhibitory antibiotic levels for less susceptible organisms [4].

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*Maxipen; Product of J. B. Roerig and Company, Div., Chas. Pfizer & Co., Inc.

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Recent in vitro studies, using laboratory cultures rather than fresh clinical isolates that are more representative of pathogenic bacteria seen in actual practice, appear to show that potassium phenethicillin is less active than other oral penicillins. Doubtlessly variation in activity in vitro favoring either this or that penicillin will be found, depending on the conditions of testing. Such variation is usually of so small a magnitude as to be clinically insignificant. A more feasible explanation of clinical effectiveness is in the measure of concentration of penicillin in the serum and tissues.

MATERIALS AND METHODS

Fifty men and fifty women, in whom the diagnosis of gonorrhea had been substantiated by smear and culture, were given oral penicillin (potassium phenethicillin) for treatment of the infection. All of these persons had presented themselves for examination as public charges at the Delgado Out-Patient Clinic, a component of the New Orleans Health Department.

To establish the most effective dosage regimen, several schedules using a 250-mg phenethicillin tablet were tried, e.g., 1 tablet t. i. d. for 6 days; 1 tablet b.i.d. for 5 days, and 1 tablet t.i.d. for 4 days.

Table 1. Summarized View of Clinical Program with Oral Potassium Phenethicillin, 250-mg Tablets B.I.D. in the Treatment of Gonorrhea in 100 Public Health Outpatients

Number of patients	Duration of treatment, days	Number of patients not returning for follow-up*	Results	
			Failures†	Complete clinical control
6	6	2	0	4
3	8	0	0	3
1	10	0	1	0
24	4	10	2	12
2	5	0	0	2
64	6	13	6	45
Total 100		25	9	66

*Presumed to be devoid of clinical symptoms.

†Patients definitely established or strongly suspected to be uncooperative; judged to be human rather than drug failures.

Patients were instructed clearly to follow faithfully the prescribed dosage regimen and to desist from all alcoholic consumption and sexual activity during the course of therapy. In addition, patients were instructed to observe themselves carefully for discharge and to note when this ceased, for painful urination, and for any unusual feelings, reactions, or sensations which might arise during the course of therapy.

Patients were asked to return for evaluation of results of treatment at the end of a week, at which time results in women were ascertained with smears and cultures obtained from the vagina, cervix, and urethra. In the males the smear and culture routine was not repeated if there was no evidence of urethral discharge, edema of the meatus, or other implicating symptoms. Patients were questioned about any possible unusual physical effects which might have occurred during treatment. All patients, upon release from treatment, were asked to return immediately if any subjective or objective evidence of a relapse occurred.

RESULTS

Twenty-five of the 100 patients did not return for their follow-up visit a week after treatment began. Of the 75 who did return for evaluation of therapy, 66 (88%) were found to be clinically free of the infection. None of the remaining nine who returned but continued to exhibit positive smears and cultures could reasonably be said to demonstrate a failure of drug therapy as close questioning revealed or raised the suspicion that the patient had not followed fully either his dosage schedule or the balance of the required program. Notably absent in this particular series of patients was any sort of skin reaction, fever, nausea, or diarrhea. One man complained of pain in his left knee while on therapy. Another stated that the medication increased his appetite for food. No sensitivity reaction was seen or reported.

COMMENTARY

It may be assumed that this new oral penicillin was successful in obtaining almost complete clinical control, or would have been had all patients properly cooperated. It may reasonably be guessed that patients who did not return for evaluation were at least symptomatically controlled. Probably, if urethral discharge or painful micturition continued the patient would have returned for further treatment. Lack of disease-control among those who did return may be attributed to human rather than drug failure. Two of these patients simply stopped taking the drug when urethral discharge ceased and therefore suffered a relapse.

Of the various dosage schedules tried, one 250-mg penicillin tablet, t.i.d. was the most effective. In about one-half of the male patients urethral discharge ceased within 12-24 hr. Most patients reported no pain on urination after 12 hr of therapy. It is interesting that most female patients, when first seen, had elevated body temperatures. Upon their return visit, their temperatures had returned to normal.

The majority of patients included in this study were persons of depressed educational and economic status. Attendance at the clinic is free. Though the oral drug is most effective, it presented the obvious difficulty for the physician of not knowing whether the patient was carefully adhering to the prescribed dosage regimen. About 10% of the patients in the study may be said to have been definitely remiss in this or other aspects of treatment. On the other hand, it should be pointed out that medical treatment has always been a problem in a small percentage of patients who do not welcome injections. Complete follow-up in all patients has never been consistently achieved, regardless of the form of medication. In the long run, it may prove possible to gain fuller cooperation of a greater number of patients with an oral penicillin regimen. With responsible patients who seek treatment for gonorrhea in a private practice, however, the physician may doubtlessly prescribe this oral penicillin with reasonable certainty that it will be properly taken.

Those treating venereal diseases, and this will or ought to include physicians in all of the medical disciplines, recognize that public health requirements necessitate rapid and complete eradication of the infection. Formerly, maximum reliability could be obtained only with parenteral penicillin forms. Potassium phenethicillin, a new oral drug, alters this situation and offers all clinicians, particularly the private practitioner, both the ease of acceptance and of administration of the oral route. In hospital and institutional settings, of course, time saved by the trained staff will be considerable if the oral drug is used. Indeed, much time was saved in our clinic by use of oral phenethicillin, in addition to which the possibility of sepsis was reduced as was the chance of occurrence of sensitization among the medical staff.

Another consideration of importance is that allergic reactions with the oral route probably occur less often and tend to be less severe if they do appear. Virtually all bacterial infections caused by penicillin-susceptible pathogens such as *Neisseria gonorrhoeae* probably should be treated via the oral route. This is not, however, even for an instant to suggest that the usual precautions regarding indiscriminate use of penicillin or the possibility of allergic manifestations be abandoned. On the contrary, under these circumstances, the physician must continue to be even more alert to these problems. Earlier efforts to minimize the allergenic properties of synthetic penicillin are to be deplored.

SUMMARY AND CONCLUSIONS

Venereal disease is on the increase, and it is the duty of all physicians, not only those doing public health work, to combat this actively through education and effective treatment. The oral penicillin (potassium phenethicillin) used in this study provided full clinical control of gonorrhea in 66 of 75 patients (88%) who were fully evaluated; it is estimated that 25 additional patients who did not return for follow-up were also fully controlled symptomatically. In some of the nine instances when treatment failed the patients admitted to not taking the drug for a full course of treatment or admitted, or were suspected, of sexual re-exposure to the disease. No allergic reactions were seen.

Other forms of oral penicillin, some doubtlessly more effective than potassium phenethicillin, are already on the clinical horizon. It can, nonetheless, be concluded now that phenethicillin represents a valuable pharmaceutical advance in the effective, safe, and convenient treatment of gonorrhea. Oral potassium phenethicillin therefore merits full clinical consideration at this time from all physicians called upon to treat gonorrhea or other infectious diseases caused by penicillin-susceptible organisms.

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THE SYNNEMATIN B SUSCEPTIBILITY OF PENICILLIN G RESISTANT GONOCOCCI

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INTRODUCTION

In the treatment of gonorrhea and syphilis, penicillin is the drug of choice. However, Brown [1] has found that untoward reactions to penicillin appear to be more prevalent and most clinics treat with other drugs those patients who claim or are known to be sensitive to penicillin.

The penicillin, synnematin B, differs from other penicillin species in that the prosthetic group is an aminoacyl structure (D-4-amino-4-carboxy-n-butyl) [2]. This individuality of structure has been shown by Berryman and Sylvester [3] to result in an antigenic function different from that of penicillin G. Thus, patients actively or passively sensitive to penicillin G (benzylpenicillin) fail to react to the injection of synnematin B.

Further evidence of the nonreactivity of synnematin B for penicillin G sensitive patients has been reported by Schwimmer and Henderson [4]. One case of syphilis and five cases of acute gonorrhea known to be sensitive to penicillin G were successfully treated with synnematin B without allergic manifestations, either early or delayed.

The effectiveness of synnematin B for acute gonorrhea of the male has been determined in 132 patients by Schwimmer et al. [5]. A single injection of 300,000 units in these bacteriologically proven cases resulted in a treatment failure rate of 4%. Synnematin B or penicillin G susceptibilities of the gonococcal strains isolated in this study were not determined.

It is well established in the United States and Europe that some gonococcal strains are relatively resistant to penicillin G and that such strains of lowered susceptibility are increasing in number [6,7,8]. It has also been found that clinical failures to penicillin therapy usually correlate with gonococcal strains of decreased penicillin susceptibility [7,8,9].

While it appears that synnematin B may be used therapeutically in penicillin G sensitive patients without regard to allergic manisfestations, it is not known whether this antibiotic will be effective against gonococcal strains relatively resistant to penicillin G.

In the present communication, the synnematin B susceptibility of gonococcal strains relatively resistant to penicillin G is presented. In addition, the in vitro killing time for gonococci has been determined for synnematin B, and its effectiveness on phagocytized gonococci in tissue culture is described.

METHOD

Fifty-four gonococcal strains were selected for in vitro susceptibility testing against synnematin B. Half (27 strains) were cultures isolated from patients unsuccessfully treated with one or more courses of repository penicillin G and ranged in susceptibility from 0.10 to 0.35 units/ml penicillin. Because of their history, these strains were designated relatively resistant. The other 27 strains were routine isolates from patients successfully treated with penicillin G, susceptible to 0.005 to 0.05 units/ml and considered highly sensitive to penicillin.

Synnematin B,* 327 units/mg potency, was dissolved in sterile distilled water and added to proteose-peptone-chocolate agar (Difco) so that the concentration range for the poured plates was 0.01, 0.05, 0.10, 0.20, 0.40, 0.60, and 0.80 units/ml.

Suspensions of the gonococcal strains in trypticase soy broth (BBL) were prepared from chocolate agar slant cultures and standardized [10] so that a 3 mm loopful contained 1000 to 3000 colony-producing units. The surfaces of the agar plates were inoculated by the streak method (8-10 streaks/plate). Control plates devoid of antibiotic were streak-inoculated with the same cultures for observation of maximum growth. After 48 hr incubation in a candle jar the plates were observed and the minimal inhibitory concentration taken as the units/ml of antibiotic that completely inhibited visible growth.

For the killing time experiments, screw-cap tubes contained 8 ml of trypticase soy broth (BBL) including synnematin B in concentrations of 10.0, 5.0, 2.5, 1.25, 0.6, 0.3, 0.15 units/ml and inoculum of 300,000 colony-producing units/ml. The tubes were incubated at 35 C rotating in a roller drum at 125 rph. At 3, 6, 9, 12, 15, 18, 24, 48, and 72 hr, 2.0 ml samples were removed. Chocolate agar plates were inoculated with 0.1 ml of the sample and the remainder added to 2.0 ml of broth containing five times the concentration of penicillinase necessary to inactivate the synnematin B. The subcultures were incubated 72 hr and observed for growth. Those tubes in which visible turbidity was not apparent were subcultured to chocolate agar plates to detect viable gonococci.

To determine the effect of synnematin B on gonococci phagocytized by tissue cells the method previously described by Thayer et al. [11, 12] was employed with the following exceptions: KB [13] instead of HeLa cells were used; infecting medium was composed of 20% fresh guinea pig serum in Eagle's medium. After phagocytosis of gonococci had occurred, the supernatant infecting medium was inactivated by adding penicillinase in trypticase soy broth. Control tubes, synnematin B in concentrations of 12.0, 6.0, 3.0, 1.5, 0.75, and 0.37 units/ml. After 48 hr contact with intra- and extracellular gonococci, the synnematin B was inactivated by adding penicillinase in Trypticase soy broth. Control tubes, devoid of tissue cells which represented extracellular conditions, were treated in the same manner as the tissue cultures. Subcultures were made at intervals on chocolate agar plates to determine the presence of viable gonococci.

RESULTS

Susceptibility testing. The synnematin B range and average minimal inhibitory concentration (MIC) for 54 gonococcal strains is shown in Table 1. Those strains considered relatively resistant to penicillin G (range 0.10-0.35 units/ml) required higher concentrations (0.4-0.6 units/ml) of synnematin B than strains highly sensitive to penicillin G. Of the relatively resistant strains, 10 had a MIC of 0.6 units/ml and 17 had a MIC of 0.4 units/ml. Among the highly sensitive penicillin G strains the MIC of synnematin B was 3 at 0.4, 12 at 0.2, 9 at 0.1, and 3 at 0.05 units/ml.

*Kindly supplied by Dr. N. D. Henderson, Michigan Department of Health, Lansing, Michigan.

Killing time. Results of the killing-time experiment are shown in Tables 2 and 3. Chocolate agar subcultures of 0.1 ml, of each synnematin B concentration tested showed that about 99.8% of the inoculum failed to grow at the 3 hr interval in concentrations of 1.25-10 units/ml. The bacteriostatic action was still more pronounced at 6 and 9 hr, and gonococci showed complete inhibition after 12 hr exposure. At concentrations below the MIC of the strain used (0.4 units/ml), growth was slowed but recovery took place at the end of 24 hr.

When 2.0-ml aliquots of synnematin B were added to penicillinase broth at the time intervals stated, gonococcide action did not take place until some time between 15 and 20 hr after exposure.

Table 1. Synnematin B Susceptibility of Penicillin G Sensitive and "Resistant" Gonococci

Number of strains	Penicillin G		Synnematin B	
	Range, units/ml	Average MIC, units/ml	Range, units/ml	Average MIC, units/ml
27	0.10-0.35	0.22	0.4-0.6	0.47
27	0.005-0.05	0.025	0.05-0.4	0.16

Table 2. Bacteriostatic Action of Synnematin B for Gonococci

Hr	Synnematin B, units/ml							
	10	5	2.5	1.25	0.6	0.3	0.15	0
3	68*	62	34	85	N	N	C	C
6	3	6	1	1	15	N	C	C
9	0	2	0	0	1	35	C	C
12	0	0	0	0	0	53	C	C
15	0	0	0	0	0	N	C	C
20	0	0	0	0	0	N	C	C
24	0	0	0	0	0	C	C	C
48	0	0	0	0	0	C	C	C
72	0	0	0	0	0	C	C	C

*Number of gonococcus colonies
N = Colonies too numerous to count
C = Colonies coalesced

Table 3. Bactericidal Action of Synnematin B for Gonococci

Hr	Synnematin B, units/ml							
	10	5	2.5	1.25	0.6	0.3	0.15	0
3	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+
15	0	+	+	∓	+	+	+	+
20	0	0	0	0	0	+	+	+
24	0	0	0	0	0	+	+	+
48	0	0	0	0	0	+	+	+
72	0	0	0	0	0	+	+	+

+ = Growth
0 = No Growth

Tissue culture. The effect of synnematin B on intra- and extracellular gonococci is shown in Table 4. After 48 hr exposure to synnematin B, gonococci were inhibited in control tubes devoid of tissue cells by 0.8 units/ml. The supernatant fluid from the infected tissue cells at this time also showed a MIC of 0.8 units/ml indicating that extracellular gonococci had been killed.

Broth containing penicillinase was then added to infected KB cells and to the control tubes. After incubation for 120 hr subcultures showed that phagocytized gonococci had been protected against the action of 6.4 units/ml of synnematin B whereas, in the absence of phagocytosis, the MIC for the gonococci remained the same, i.e., 0.8 units/ml.

Table 4. Action of Synnematin B on Intracellular and Extracellular Gonococci

Synnematin B, units/ml	Intracellular (KB cells)		Extracellular (Eagle's medium)	
	48-hr culture	120-hr* subculture	48-hr culture	120-hr* subculture
12.8	0	0	0	0
6.4	0	+	0	0
3.2	0	+	0	0
1.6	0	+	0	0
0.8	0	+	0	0
0.4	+	+	+	+
0	+	+	+	+

*Penicillinase added to all tubes
0 = No growth
+ = Growth

DISCUSSION

Synnematin B and penicillin G minimal inhibitory concentrations for gonococci cannot be compared on the basis of the antibacterial unit because standardization methods differ. A unit of sodium synnematin B is about equal to 1.4 μg of pure antibiotic while a unit of penicillin G represents 0.6 μg [14]. However, gonococcal strains relatively resistant to penicillin G required higher concentrations of synnematin B than did those strains which were highly sensitive to penicillin G. Gonococcal strains relatively resistant to penicillin G amount to about 20% of the routine cultures examined for most parts of the United States.

While within 3 to 6 hr the greater portion (99.8%) of gonococci are inhibited by in vitro exposure to synnematin B, bactericidal action is not complete until some time between 15 and 20 hr. Similar results have been obtained with penicillin G. However, bactericidal action for some strains does not occur until between 24 and 36 hr exposure [6].

The presence of such "persistors" in vivo might account for treatment failure in the absence of sufficient tissue concentrations of the antibiotic.

Sylvester, et al. [15], report that the average blood levels developed at 3 hr by patients treated with 200,000 and 400,000 units of aqueous synnematin B are from 3.5 to 6.5 units/ml respectively, and 0.7 to 1.5 units/ml at the end of 8 hr. Blood levels beyond 8 hr were not determined. It would seem desirable to treat patients with multiple injections of the antibiotic in order to have adequate tissue concentrations and to prevent the development of synnematin B resistance such as has occurred with penicillin.

Synnematin B is like penicillin in that intracellular gonococci are protected against its action in tissue culture experiment. Bang [16] has observed gonococci within columnar epithelial cells of the human host and for this reason more intensive treatment may also be indicated, particularly in the female where numerous glandular structures may lend themselves to intracellular infections.

CONCLUSIONS

1. Relatively resistant penicillin G gonococci require higher concentrations of synnematin B to inhibit in vitro growth than do strains highly susceptible to penicillin G.
2. Gonococci are rapidly killed in vitro within the first 9 hr of exposure to synnematin B, but bactericidal action is not complete until between 15 and 20 hr.
3. Gonococci phagocytized by tissue cells in vitro are protected against the action of synnematin B.

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EARLY RESULTS IN THE TREATMENT OF PRIMARY AND SECONDARY SYPHILIS WITH POTASSIUM PHENETHICILLIN (MAXIPEN)*

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INTRODUCTION

The guiding principle in the antibiotic treatment of syphilis is the maintenance of a treponemicidal blood level for approximately 10 days in early syphilis and 15-20 days in late syphilis. This is as true with penicillin [1] as with the so-called broad-spectrum antibiotics [2]. Passage through and absorption from the gastrointestinal tract generally produces a lower blood level than the comparable quantity of the agent given intramuscularly [3]. Also, the oral preparations maintain the blood level for a shorter period of time and this requires more frequent dosage with increased patient cooperation. For these reasons, and its as yet unsurpassed treponemicidal action and lower cost, preparations of long-acting penicillin given intramuscularly remain the treatment of choice in syphilis. However, there are occasions when penicillin by injection is not desirable, where cost and patient reliability are not factors. Confirmed penicillin-sensitive patients, as well as those unable to receive periodic injections might require alternate therapeutic regimes of an oral antibiotic. It was with this group in mind that we undertook to evaluate the efficacy of potassium phenethicillin in the treatment of early syphilis.

SELECTION OF STUDY CASES

Patients admitted to the San Francisco City Clinic with darkfield-positive lesions of primary and secondary syphilis and who appeared reliable in terms of taking the medication as prescribed and returning for follow-up were considered eligible for the study. Serologic tests for syphilis (STS) were also taken at time of diagnosis, but their results did not influence selection of study cases. Twenty-seven patients (26 males and 1 female) were then started on a course of treatment which consisted of 500 mg of potassium phenethicillin q.i.d. for 10 days. Theoretically, giving the medicine every 6 hr would be more desirable, but such a goal with the majority of people, and our clinic population in particular, is unrealistic. At completion of therapy, 10 days later, clinical evaluation of the lesions and history of reaction was secured. STS were repeated 4-6 weeks later.

RESULTS AND DISCUSSION

Of the 27 patients started on the treatment schedule, 25 completed it (Table 1). Patient No. 14, the lone female in the series, discontinued treatment as a gesture of marital defiance. This case cannot be considered a therapeutic failure or unfavorable side reaction due solely to the drug. Patient No. 15 had a history of previous penicillin allergy. Hoping the allergenic composition of this synthetic

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*The drug used in this test was supplied by J. B. Roerig and Co., a division of Chas. Pfizer and Co.

Table 1. Early Results in the Treatment of Primary and Secondary Syphilis with Potassium Phenethicillin

No.	Diagnosis	Initial STS (VDRL in dils.)	STS after treatment (4-6 weeks)	History of penicillin allergy	Herxheimer reaction	Other reaction
1	Pri	R 8	WR	–	–	–
2	Pri	R 64	R 32	+	–	–
3	Pri	R 0	NR	–	–	–
4	Pri	NR	NR	–	–	–
5	Sec	R 32	R 4	–	+	–
6	Pri	R 8	R 0	–	+	–
7	Pri	R 0	R 0	–	+	–
8	Pri	NR	NR	–	+	–
9	Pri	NR	WR*	–	–	–
10	Pri	R 0	NR	–	–	–
11	Sec	R 16	R 4	–	–	+[1]
12	Pri	NR	NR	–	–	–
13	Pri	NR	NR	–	+	–
14	Pri	R 16	R 2	–	–	–[2]
15	Pri	R 2	NR	+	+	+[3]
16	Pri	NR	R 2*	–	+	–
17	Pri	NR	NR	–	+	–
18	Pri	WR	NR	–	–	+[4]
19	Pri	NR	NR	–	–	–
20	Pri	NR	NR	–	–	–
21	Pri	R 8	R 0	–	+	+[5]
22	Pri	R 2	NR	–	–	–
23	Pri	R 32	R 16	–	+	–
24	Pri	NR	NR	–	–	–
25	Pri	NR	NR	–	–	–
26	Sec	R 64	R 8	–	–	–
27	Sec	R 32	R 32	–	+	–

R = Reactive
WR = Weakly reactive
NR = Nonreactive

*Next STS (4-6 weeks later) NR.

1. Diarrhea first day of treatment.
2. Patient stopped treatment after seventh day for nonmedical reasons. Finished with penicillin IM.
3. Developed rash after first day treatment; becoming highly pruritic (second day) and treatment discontinued. Finished treatment with oral tetracycline.
4. Mild gastrointestinal upset, loss of appetite.
5. Diarrhea first few days of treatment.

penicillin was sufficiently different, and knowing the medication could be discontinued and quickly metabolized, we felt use of the drug worthwhile. Unfortunately, rapid development of a pruritic rash forced discontinuance of the agent. The remaining 25 finished the course of therapy as prescribed. All improved clinically with involution of body rashes and healing of ulcers by the tenth day of treatment. Patients No. 11, No. 18, and No. 21 had mild gastrointestinal disturbances during the course of therapy which spontaneously subsided despite continuance of the drug. As only three cases were involved, it is difficult to evaluate the causative relationships.

Circulating serum antibody (reagin) in very early syphilitic infections is frequently below the measurable level determined by the standard serologic tests for syphilis. This explains the nonreactive results in 11 of the 21 cases of primary infection. In two of these (patients No. 9 and No. 16), it would seem that the impetus of reagin formation continued after treatment was started, producing sufficient antibody to show as a reactive STS at the time of the first posttreatment follow-up examination. Both had nonreactive tests 4-6 weeks later. This is a relatively common phenomenon, occurring with the best known treatment regimes. Of the 14 patients with STS's showing some degree of reactivity, 12 showed a drop by the time of the first follow-up examination. Continued reagin formation as described above, or individual variations allowing a slight degree of sero-resistance, explain the two cases which remained at previous levels. Such serologic results represent clear examples of why single test reports are not in themselves diagnostic, but must be related to history.

In the clinical trial of new drugs, the researcher must evaluate side reactions as possible toxic manifestations. When the new agent is used in the treatment of syphilis, the Herxheimer reaction may cloud the picture. In observing or taking a history from the syphilitic patient for possible toxic reactions, the researcher learns of signs and symptoms which he attributes to the Herxheimer pattern. These frequently are vague and transient. Depending upon the criteria of diagnosis previously established and the diligence of exploration into this area of clinical trial, the number of Herxheimer reactions reported incidental to studies of syphilis therapy may vary tremendously [4]. The physician may be influenced by older concepts of so-called "therapeutic shock" and "therapeutic paradox" [5] and may feel that a high incidence of the reaction is something to avoid. Although varied theories of its cause exist, it is common to all effective treponemicidal agents. Current thought [6, 7] is that it should not affect choice of therapeutic regime, certainly not in primary and secondary syphilis.

CONCLUSION

While it is not possible to evaluate antisyphilitic drugs definitively on a short-term basis, reporting of initial satisfactory clinical and serologic response with absence of toxic manifestations has some value. Under these terms, potassium phenethicillin, in dosage given, appears to be comparable to the most effective antisyphilitic regime extant. The drug warrants close and continued appraisal.

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ORAL PHENOXYETHYLPENICILLIN (MAXIPEN)* IN THE MANAGEMENT OF SUBACUTE BACTERIAL ENDOCARDITIS AND OTHER SEVERE INFECTIONS

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INTRODUCTION

The variable and relatively low serum levels obtained after the ingestion of penicillin G tablets [1] made the oral approach to adequate penicillin therapy impractical for moderately severe infections. The efficiency of penicillin V absorption and the higher and more sustained blood levels [2–4] over penicillin G following comparable doses made its therapeutic trial in Gram-positive infections mandatory.

Batchelor et al. [5] in 1959 isolated 6-aminopenicillanic acid. This led to the development of a new synthetic product, more commonly known as α-phenoxyethylpenicillin. At the time of the initiation of our investigation the report of Knedsen and Rolinson [6] revealed that a 250-mg tablet of phenoxyethylpenicillin produced serum levels twice that of penicillin V and equal to those obtained with 400,000 units of intramuscularly administered crystalline penicillin G. I might add that some other views on this subject have been expressed more recently [7, 8].

DISCUSSION

A study was undertaken to determine the clinical effectiveness and toxicity of phenoxyethlypenicillin in a variety of moderate and severe penicillin-sensitive infections. One-hundred patients were evaluated (Table 1).

Subacute bacterial endocarditis. Four years ago Quinn et al. [9, 10] reported the results of oral penicillin V, combined in most instances with streptomycin, in eleven cases of subacute bacterial endocarditis, Ten of the eleven patients were treated successfully. Additional case reports have been reviewed in the literature [11-15] and the success of oral penicillin therapy in some eighteen cases of subacute bacterial endocarditis is well documented. It is apparent that the best results were obtained when the alpha-hemolytic streptococcus was the etiologic agent.

Eleven cases of subacute bacterial endocarditis were managed with oral phenoxyethlypenicillin. The organisms cultured from the blood in eight cases are listed in Table 2. Three patients were treated for subacute bacterial endocarditis despite negative blood cultures. Each case received 1500 mg (6 tablets) of phenoxyethlypenicillin every 4 or 6 hr before meals, for a minimum of four weeks. One hour penicillin levels ranged from 10 to 25 μg/ml; 4-hr serum levels from 5 to 11μg/ml; and 6-hr levels up to 2.11 μg/ml. There were no fatalities in this series, although one patient experienced a cerebral embolus during the sixth

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*Phenoxyethylpenicillin supplied by J. B. Roerig & Co. Division, Charles Pfizer & Co., Inc., Brooklyn, New York.

Table 1. Diagnosis of 100 Cases Treated with Phenoxyethylpenicillin

Infections treated	Number of cases
Pneumonia	38
Tonsillitis	21
Subacute bacterial endocarditis	11
Bronchitis	11
Furunculosis	3
Sinusitis	3
Pyelonephritis	3
Thrombophlebitis	2
Tracheobronchitis	1
Osteomyelitis	1
Soft tissue abscess	1
Pyodermia	1
Central nervous system syphilis	1
Prostatitis	1
Pyogenic arthritis	1
Empyema	1

Table 2. Data on 11 Cases of Subacute Bacterial Endocarditis

Organism	Number of cases
Alpha-hemolytic streptococci	4
Staphylococcus aureus	2
Gamma streptococci	1
Anaerobic streptococci	1
Negative blood culture	3
Bacteriologic cures	8
Clinical cures	10
Complications (cerebral embolus)	1
Deaths	0

week of oral penicillin therapy. Blood cultures in this case initially revealed an anaerobic Streptococcus and all interval cultures after the first week of therapy remained negative. The two following cases illustrate treatment.

Case 1. A 58-year-old white female was admitted to the hospital with a one-month history of palpitation, weakness, tremors, and anorexia. The symptoms followed a vaginal plastic operation. Physical examination noted a chronically ill female with fine crepitant rales at both lung bases, atrial fibrillation, and a grade two systolic murmur at the apex. Chills and temperature up to 101 F were encountered soon after admission and four blood cultures were positive for alpha-hemolytic Streptococcus. The organism was highly sensitive to penicillin, as shown by tube dilution studies. Initial therapy consisted of 8-million units penicillin and 1 g streptomycin/day introduced intramuscularly. After five days of therapy, intramuscular medications were discontinued due to severe reactions at the site of injection. Phenoxyethylpenicillin, 1500 mg every 6 hr, was substituted. The patient became afebrile and serum inhibition studies against the alpha Streptococcus isolated on admission revealed a bactericidal effect in a 1:128 dilution. Oral therapy was continued for five weeks and the patient made an uneventful recovery. Interval blood cultures remained negative and the patient remained asymptomatic.

Case 2. A 58-year-old white male was admitted to the hospital with a two month history of weakness, anorexia, and two week history of hematuria, chills, and fever. Past history was noncontributory. Physical examination revealed a temperature of 103 F and a grade three harsh systolic murmur at the apex which radiated to the left axilla. The white blood count was 14,800, hemoglobin 11 g/100 ml, and the urine contained 8–10 red blood cells in a high-power field. Blood cultures, on admission, revealed alpha-hemolytic streptococci. Therapy consisted of phenoxyethylpenicillin, 1000 mg (4 tablets) every 4 hr and serum inhibition studies revealed a bactericidal effect in a 1:32 dilution. The patient made an uneventful recovery in six weeks.

We have all been faced with the situation of an apparently clear cut case of endocarditis only to find repeatedly negative blood cultures. In most instances antibiotics had been administered prior to hospitalization and before the initial blood cultures were obtained. Three such cases were managed with oral penicillin. Case No. 3 serves as an example.

Case 3. A 64-year-old white male with rheumatic heart disease and aortic insufficiency was admitted with shortness of breath, productive cough, weight loss, and fever of several weeks duration. Past history revealed two previous allergic (skin) reactions to parenteral penicillin. Admission temperature was 102 F. A loud systolic murmur was heard over the aortic area and splenomegaly was present. Numerous blood cultures were negative. The patient was treated for subacute bacterial endocarditis with 1500 mg (6 tablets) phenoxyethylpenicillin every 6 hr after an initial test dose revealed no toxic or allergic reactions. Therapy was continued for six weeks and the patient's subsequent course was uncomplicated with complete relief of all symptoms.

The choice of oral antibiotic therapy avoids repeated painful injections and intravenous infusions. It permits a more practical method for continuous therapy in both hospitalized and nonhospitalized patients. Our experience and those of others, confirm its clinical efficacy.

The case of alpha-hemolytic streptococcal endocarditis, highly sensitive to penicillin, exhibiting neither acute heart failure nor embolic phenomena, would appear to be the ideal candidate for oral synthetic penicillin therapy.

Pneumonia. Hart et al. [16] and McWhorter et al. [17] previously demonstrated the effectiveness of oral penicillin V in pneumonia. Austrian and Winston [18] successfully treated 71 of 73 patients with pneumococcal pneumonia with 400,000 units of oral penicillin V every 12 hr. Reeves et al. [19] treated 145 cases with 250 mg penicillin V every 6 hr, and observed satisfactory results in 95% of the cases.

Thirty-eight cases of pneumonia, twenty-two pneumococcal, were treated without a single failure. The dosage of phenoxyethylpenicillin varied but the ma-

Table 3. Phenoxypenicillin Therapy of 38 Cases of Pneumonia

Dosage	Number of cases
1 tablet q.i.d. or q. 6 hr	1
2 tablets q. 6 hr	27
3 tablets q. 6 hr	1
4 tablets q. 6 hr	7
6 tablets q. 6 hr	2
Duration of therapy, days	
1-6	9
7-13	23
14-20	5
21-27	1
Response to clinical therapy	
24 hours or less	19
48 hours	6
72 hours	6
4-6 days	6
7-13 days	1

jority of cases received 500 mg (2 tablets) every 6 hr (Table 3). Thirty-three patients received oral penicillin alone while four received in addition streptomycin and one tetracycline. Duration of therapy ranged from 1 to 13 days in 32 patients and satisfactory clinical response was noted in 80% of the cases within 72 hr.

Case 4. A typical case was that of a 42-year-old Negro female who was admitted to the hospital with a two week history of cough and a one day history of left pleural chest pain, rusty sputum, chills, and fever. Physical examination revealed a temperature of 103 F, dullness to percussion, decreased breath sounds, and subcrepitant rales at the left lung base. Chest x-ray confirmed the left lower lobe pneumonia, and sputum and blood culture revealed Diplococcus pneumoniae. The patient was treated with 500 mg (2 tablets) phenoxyethylpenicillin every 6 hr. She became afebrile in 12 hr and she exhibited rapid clinical and radiographic improvement.

Other serious infections. Time does not permit a full discussion of a variety of penicillin-sensitive infections treated with phenoxyethylpenicillin (Table 1). The average daily schedule was 1000 - 2000 mg given in 3 or 4 divided doses. A favorable clinical or bacteriologic response was noted in all cases. Particularly noteworthy are the cases of alpha-streptococcal arthritis, enterococcal pyelonephritis, and staphylococcal prostatitis and osteomyelitis.

COMMENT

There were no allergic reactions recorded in the 100 patients treated with α-phenoxyethylpenicillin. However, others have indicated cases of skin sensitivity reactions [20]. There was one episode of nausea and vomiting and four patients experienced transient diarrhea. There was not a single instance of gastrointestinal disturbance in the eleven patients treated for subacute bacterial endocarditis with 24-36 tablets/day.

In summary, 100 cases of moderate and severe infections have been treated with oral phenoxyethylpenicillin with consistent therapeutic success. The study included 11 cases of subacute bacterial endocarditis and 38 cases of pneumonia.

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DISCUSSION

DR. KIRBY (Seattle, Washington): I would like to congratulate you, Doctor Gold, on this paper.

DR. ROMANSKY (Washington, D.C.): I'd like to make one point in connection with patients with negative blood cultures. In the past two years, we have had an extremely unusual experience in a patient who presented a classic subacute bacterial endocarditis with negative blood cultures. He was treated and fifteen months later had to be treated again, but all blood cultures were still negative. Subsequently, the patient was seen at another institution in the midwest and inadvertently the blood cultures were all left at room temperature one night and they all grew out with a staphylococcus extremely sensitive to penicillin, a very peculiar organism which would only grow at room temperature. I would suggest, therefore, that in the presence of negative blood cultures, it would do well to request the laboratories to try their cultures at varying temperatures as well as the standard incubator temperature.

I think that the fact that the organism grew at room temperature and not at 37 C played a part in the infection.

I don't know the exact mechanism, but it is probable that the organism was driven into the tissues or somewhere and then subsequently it reappeared. It hardly seems likely that there were three reinfections (I mean reinfection other than relapse).

CLINICAL EVALUATION OF DIMETHOXYPHENYLPENICILLIN*

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INTRODUCTION

The treatment of patients with infections caused by penicillinase-producing staphylococci has become an increasingly difficult and serious problem during the past fifteen years. The effectiveness of most antibiotics introduced during this time has been limited by the development of resistant organisms or by side effects. Vancomycin, although it is a potent bactericidal antibiotic which can be given in doses adequate to produce high blood and tissue levels and to which staphylococcal resistance has not appeared [1], has been restricted to hospitalized patients with severe infections because of the necessity of intravenous administration and the occurrence of phlebitis, chills, and fever in a significant percentage of cases. Kanamycin, neomycin, ristocetin, and bacitracin have had their usefulness markedly restricted by significant toxicity or side effects.

The isolation by Batchelor [2] in 1959 of 6-aminopenicillanic acid drew attention to the possibility of synthesizing new forms of penicillin by the introduction of suitable side-chains. One such derivative, dimethoxyphenylpenicillin,† has shown marked resistance to staphylococcal penicillinase in vitro, and can be given in amounts adequate to produce high serum concentrations with few side effects. Staphylococcal resistance has developed relatively slowly in vitro, and no resistance has been observed so far in patients. This report presents the results of therapy in 44 patients treated with dimethoxyphenylpenicillin during a four-month period.

METHODS AND MATERIALS

The 44 patients in this study were treated during the period June, 1960-October, 1960 in the University of Washington Affiliated Hospitals. All cases were seen frequently by one of the authors. Patients with staphylococcal infections were treated if their infection was clinically significant and if they gave no history of penicillin allergy. In addition, during the early part of this study, most patients at the Kings County Hospital admitted with the diagnosis of Gram-positive bacterial pneumonia were treated with this drug.

Dimethoxyphenylpenicillin was supplied as a powder; for intramuscular injection, 1.6 ml of sterile water was added to each 900-mg ampoule and the resultant 2 ml of solution injected immediately. For intravenous injection each 900-mg ampoule was diluted in 15 ml of normal saline solution and injected slowly over 5 min to minimize venous irritation.

Most patients were treated with 900 mg every 4 hr intramuscularly and the interval was increased in some cases to every 6 hr after signs of clinical improvement occurred. Only four patients received larger doses intramuscularly, of 1.8 g every 4 hr, and this was attended by somewhat more discomfort in the injection site. A course of therapy usually lasted 5-7 days and consisted of 20 to 30 g. Some patients with particularly severe or chronic infections, such as

*The new generic name for dimethoxyphenylpenicillin is methicillin.

†Kindly supplied as Staphcillin by Bristol Laboratories, Syracuse, New York.

osteomyelitis, received as much as 270 g over 34 days. Probenecid, 2 g daily, was given routinely to those patients able to take oral medication. Four patients received other antibiotics while receiving dimethoxyphenylpenicillin, usually because of the coexistence of a Gram-negative infection.

Attempts to add the drug to intravenous infusion bottles resulted in gross precipitation at the end of 4-6 hr, with loss of antistaphylococcal activity. This appeared related to the acid pH (under 5) of standard intravenous solutions. However, when the pH of a liter of 5% dextrose solution was buffered to 7.2 by the addition of 3 meq of sterile sodium bicarbonate, the drug was stable for more than 24 hr. Using this technique, three patients were treated with constant intravenous infusions, receiving 900 mg hourly, in an attempt to assess possible toxicity and to determine serum levels.

RESULTS

Of the 44 patients treated with dimethoxyphenylpenicillin, 26 had significant staphylococcal infections, 11 had pneumococcal pneumonia, 2 had streptococcal cellulitis, and 5 others had an assortment of infections caused by Gram-negative rods or noninfectious diseases. A summary of the response of these 44 patients to therapy is presented in Table 1.

Table 1. Results of Therapy in Forty-Four Patients Treated with Dimethoxyphenylpenicillin

Diagnosis	Total	Cured	Improved	Unimproved
Nonstaphylococcal diseases				
Pneumococcal pneumonia	11	11		
Streptococcal cellulitis	2	2		
Gram-negative infections and noninfectious diseases	5			5
Staphylococcal infections				
Pneumonia	5	3	1	1
Septicemia	4	4		
Osteomyelitis, acute	2	1	1	
Osteomyelitis, chronic	4	2	2	
Soft tissue infections				
Deep or multiple superficial abscesses	7	3	4	
Parotitis	2	2		
Bronchitis	1		1	
Empyema	1			1
Total	44	28	9	7

Nonstaphylococcal diseases. The 11 patients with pneumococcal pneumonia were mostly adults either in the older age groups or were younger debilitated alcoholics. One was a child and she received other antibiotics concurrently because no definite organism could be identified from sputum cultures. All 11 patients were cured and showed a definite decrease in toxicity, fever, cough, and sputum within 48 hr. Nine were afebrile in five days although one debilitated alcoholic and an 85-year-old man with a sterile pleural effusion and chronic lymphatic leukemia each required twelve days to become afebrile. Ten patients received 3.6-5.4 g daily for five days only. Past experience has demonstrated that, for most patients, five days of penicillin therapy is adequate for the treatment of pneumococcal pneumonia [3].

The two patients with streptococcal cellulitis had widespread involvement of a lower leg; both became afebrile and were cured within six days on therapy with 3.6 g daily. The five remaining patients had a variety of Gram-negative or noninfectious conditions, including tuberculosis, sarcoidosis, cat scratch fever, and pneumonia caused by Gram-negative rods, and appeared unchanged by therapy.

Staphylococcal infections. Of these 26 patients, 15 were cured, 9 were improved, and 2 were not improved by therapy. Five patients were hospitalized primarily because of cancer or leukemia, and three others due to advanced arteriosclerosis. Two of the latter group died of the underlying diseases following apparent cure of the staphylococcal infections. Six patients had one or more positive blood cultures prior to therapy and all were cured.

Five patients with staphylococcal pneumonia were treated with 8- to 16-day courses of dimethoxyphenylpenicillin. Three were cured and one was definitely improved by therapy. One of these patients, a nine-year-old girl with two positive blood cultures and staphylococcal pneumonia, had become increasingly ill with the development of anemia, leukopenia, and an increasing number of lung abscesses during courses of penicillin, chloramphenicol, ristocetin, furaltadone, and oleandomycin therapy. She improved dramatically and was cured after 16 days of 5.4 g of dimethoxyphenylpenicillin daily. Another patient, a 37-year-old man with metastatic carcinoma of the rectum, developed a severe postoperative staphylococcal pneumonia but was well enough to be discharged home after nine days of 5.4 g of dimethoxyphenylpenicillin daily. The remaining patient was an old man who remained febrile through eight days of therapy and developed a lung abscess in the area of pneumonitis. This may have been an aspiration lung abscess, although staphylococci were cultured repeatedly from tracheal aspirates. His fever subsided, and clinical resolution promptly followed change to chloramphenicol therapy. He is thus classified as unimproved.

The four patients with septicemia were all cured by 11-21 days of therapy with dimethoxyphenylpenicillin. Two were children. One with leukemia and high spiking fevers had a beginning perirectal abscess and a positive blood culture after therapy with penicillin, but became afebrile and his perirectal induration resolved after 17 days of dimethoxyphenylpenicillin. The other child, a $2\frac{1}{2}$-year-old girl with a noncommunicating hydrocephalus and a ventriculo-atrial shunt, had three blood cultures positive for a penicillin-resistant *Staphylococcus albus*, and was cured after 11 days of intramuscular therapy with 5.4 g daily and a revision of her shunt.

The remaining two patients were adults. One, an 82-year-old man, developed temperatures to 104 F and two positive blood cultures for *S. aureus* while on chloramphenicol therapy following surgery for carcinoma of the bladder. He became afebrile after receiving dimethoxyphenylpenicillin for four days and appeared cured after 12 days of therapy. The other adult, a 46-year-old ex-narcotic addict, was hospitalized with temperatures to 104 F despite chloramphenicol therapy, and subsequently had five blood cultures positive for a penicillin-resistant *S. aureus*. No primary focus was found although septic pelvic thrombophlebitis was considered most likely. He became afebrile in three days and there was a remarkable return of physical vigor after five days of therapy with 1.8 g intramuscularly every 4 hr. He was then treated an additional 16 days with 1.8 g of the drug every 6 hr and appeared to be cured.

Two patients with acute and four patients with chronic osteomyelitis were treated and results appeared quite favorable. Of the two with acute osteomyelitis, one was a 14-year-old boy with severe pain in the sacroiliac area, high fever, and two blood cultures positive for a penicillin-resistant S. aureus, developing a week after a fall onto his low back. He received 20 days of therapy with 5.4 g daily and then appeared cured. On the day after therapy was discontinued he developed a typical serum-sickness type allergic reaction which cleared during the next four days. The other patient received 14 days of therapy for acute osteomyelitis of the tibia and appeared to be cured but has since developed a relapse in the same area. In this instance more prolonged therapy might have given a better result.

Four patients with chronic osteomyelitis were treated, three in conjunction with extensive surgical debridement of deep draining lesions of the pelvis or spine, the fourth in association with amputation of a chronically infected foot and ankle. The three who had debridement received 14- to 34-day courses of drug therapy and, although the follow-up period is still short, relapses have not occurred.

Of eight patients with soft tissue infections, two had large carbuncles, two had deep paraspinal abscesses, and three had recurrent furunculosis. Three were cured and the remainder markedly improved although the organism could still be cultured from the healing wound when therapy was stopped. Two patients with staphylococcal parotitis were both in their ninth decade; one had had extensive abdominal surgery and the other was extremely debilitated from malnutrition and senility. Both patients responded well and were considered cured after 7- and 12-day courses of therapy, although the second later died of problems related to advanced arteriosclerosis.

Of the remaining two patients, one with staphylococcal bronchitis was improved on therapy and had become afebrile when she suffered a fatal myocardial infarct. A 47-year-old man developed a staphylococcal empyema following an emergency thoracotomy for cardiac arrest. He improved initially with decreased drainage from his chest. Gram-negative bacteria then appeared in the drainage fluid and staphylococci were still present after 11 days. Other antibiotics were given and drainage eventually ceased. He was classified as unimproved.

TOXICITY AND SIDE EFFECTS

There was remarkable freedom from toxicity and side effects among these patients. Patients were able to tolerate a 2 or 3 ml injection containing 0.9 or 1.8 g of this drug with moderate discomfort consisting of local muscle pain which usually subsided within 10 min. One patient received over 70 injections in his deltoids alone over a 14-day period and another took over 100 injections in one gluteal area over a 20-day period. Pain occurred, but in both cases the drug was surprisingly well tolerated with mild induration at the injection sites which subsided within a week or two after cessation of therapy.

One allergic response occurred in this series. This was a moderately severe serum-sickness type reaction after 20 days of therapy which subsided after four days. It has been noted [5] that allergic reactions to this preparation are similar to those which occur with penicillin G. One patient developed a superficial skin ulcer which may have been related to injection of the drug into subcutaneous tissues. Two patients who received the drug intravenously noted anorexia and a "salty" taste in their mouths, although this did not necessitate stopping therapy.

Two patients with impaired renal function (blood urea nitrogen of 33 mg% in both) were given 900 mg of the drug hourly in a constant intravenous infusion. Serum concentrations of dimethoxyphenylpenicillin stabilized at 320 μg/ml, roughly 20 times the peak levels obtained with intramuscular injections of 900 mg, and the patients exhibited no signs or symptoms of toxicity. Of importance in relation to the therapy of cardiacs, it should be noted that each 900-mg ampoule contains 2.5 meq of sodium and that the usual daily dose of 5.4 g is thus roughly equal to 800 mg of sodium chloride.

DISCUSSION

It is evident from these results that dimethoxyphenylpenicillin is an effective antibiotic in the therapy of infections due to Gram-positive cocci. Particularly impressive were the results in the 26 patients with staphylococcal infections since 24 were caused by penicillinase-producing bacteria highly resistant to the action of penicillin G. The fact that all six of the patients with positive blood cultures were cured is impressive but is not of definitive value from a comparative standpoint since the number of cases is so small. In general, the clinical results in these patients with staphylococcal infections seem comparable to those we have accumulated over the past few years with vancomycin.

Our laboratory studies of dimethoxyphenylpenicillin are published elsewhere [4], and are in accord with the results of others [5, 6, 7]. Briefly, dimethoxyphenylpenicillin has an inhibitory and lethal action against penicillinase-producing staphylococci in concentrations ranging from 2 to 5 μg/ml. There is little if any inactivation by penicillinase, and resistance to the drug develops slowly in vitro, at about the same rate as with penicillin G. An intramuscular injection of 900 mg of the antibiotic gives a peak serum level of 12-15 μg/ml at 1 hr, with a rapid decline to less than 2 μg/ml, 4 hr later. Thus, the ratio between the minimum inhibitory concentration (2-5 μg/ml) and the peak blood level of 12-15 μg/ml is quite favorable, although it is quite possible that higher blood levels are desirable for severe infections. In some instances, 1.8 g was injected intramuscularly every 4 or 6 hr, a regimen that was quite well tolerated. Also, with appropriate adjustment of the pH of intravenous fluids as described in the text, it is possible to administer large amounts of the antibiotic in a continuous intravenous infusion without precipitation or rapid loss of potency.

One patient in this series developed an allergic penicillin reaction and it is apparent that the problem of hypersensitivity will be present, as it is with the other penicillins. In four patients staphylococci cultured after a week or more of therapy remained fully susceptible to the new antibiotic. Dimethoxyphenylpenicillin is quite well tolerated when injected intramuscularly but because of rapid excretion has the disadvantages of the early penicillin G preparations of requiring administration every 4-6 hr. Vancomycin, another potent antistaphylococcal drug, can be administered intravenously every 12 hr because of slower excretion, but has the disadvantages of causing venous irritation and of producing chills and fever in an appreciable percentage of cases. Dimethoxyphenylpenicillin is probably useful solely for the treatment of staphylococcal infections. Vancomycin, on the other hand, also has a role in the therapy of certain streptococcal infections, chiefly those due to enterococci.

SUMMARY

Forty-four patients were treated with a new semisynthetic penicillin, dimethoxyphenylpenicillin. Of 26 patients with staphylococcal infections, mostly

due to penicillinase-producers, 15 were cured, nine improved, and two unimproved. All of the six patients with blood cultures positive for staphylococci were cured. It was possible in four cases to isolate staphylococci after more than seven days of therapy, and in each instance the organism remained susceptible to the antibiotic. Results in the 18 patients with nonstaphylococcal diseases were comparable to those that might be expected with penicillin G.

One of the chief limitations of this drug is its extremely rapid excretion, requiring intramuscular injections every 4-6 hr. It is not absorbed orally, and for continuous intravenous administration it is necessary to adjust the pH of the infusion fluids. Intramuscular injections were in general well tolerated, without undue pain or induration at the injection sites. One patient developed a serum sickness type allergic reaction and it appears that the problem of hypersensitivity will be present, as it is with other penicillins.

Dimethoxyphenylpenicillin gives promise of being a safe and effective agent for the therapy of penicillin-resistant staphylococcal infections. It would appear to have no advantages in infections caused by other organisms.

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DISCUSSION

DR. RUTENBERG (Boston): I'd like to compliment Dr. Allen on a superb presentation, particularly on the clarity of his slides. We have had a similar experience with 2,6-dimethoxyphenylpenicillin in the treatment of about 60 or 70 patients with a variety of staphylococcal infections. These included primary soft tissue infections, postoperative wound infections, infected burns, pneumonias, cholangitis, parotitis, and two patients with meningitis, and about 15 with postoperative staphylococcal pneumonia. In all instances the response to the drug was very satisfactory and staphylococci were generally promptly eliminated from the infected sites and the blood stream or other sites where infection was localized. There are three observations which I would like to make. First of all, whereas the staphylococci were very promptly eliminated, and by promptly I means within 48-72 hr, Gram-negative bacteria or enterococci, which in some instances were also present in the infected nidus, were not eliminated and subsequently other antibiotics were required in some instances to suppress the other species. Secondly, in all instances, wherever it was indicated, appropriate surgical management of the wound was carried out in conjunction with chemotherapy. Now chemotherapy was indicated in the patient with soft tissue infections or postoperative wound infections because of uncontrolled spreading infection and a severe systemic response. It was not just a question of a localized carbuncle or localized wound infection which, as we well know, respond well to prompt incision and drainage, these were patients who were clinically ill with fever, leukocytosis, anorexia, malaise, and other usual signs and symptoms of a severe systemic reaction.

And in those patients, in addition to chemotherapy, appropriate surgical management of the wound or of the localized infection was carried out, such as drainage of an abscess, debridement of an infected wound, removal of a foreign body, closure of perforated ischus, and so forth. In addition to that (this, of course, is a very preliminary observation in a relatively small group of patients) three of the patients had known an observable sensitivity reaction to penicillin G. All three patients who were critically ill due to staphylococcal infections and had failed to respond to a variety of other antibiotics, were given 2,6-dimethoxyphenylpenicillin without a reaction. Then again, we had two patients who had not had penicillin G or 2,6-dimethoxyphenylpenicillin previously but they did have a reaction in the course of therapy after about four or five days. They developed a skin reaction and a rash which was very similar to the type that we observe in penicillin G sensitive patients. Apparently this drug can cause allergic reactions but there does not seem to be any evidence of cross sensitization with penicillin G.

DR. ROMANSKY: In the early days of penicillin, 1943-46, at Walter Reed, several of the patients that we treated developed allergic reactions but several months later we again gave them penicillin G and then there were no reactions. So, although what you say may be valid, I don't think it's proven by the statement that they were able to take the dimethoxypenicillin without developing a rash, and I don't think this necessarily holds in an over-all fashion.

DR. ALLEN: I might just say that Stewart, in a recent issue of the British Medical Journal, has reported two patients who had previous sensitivity to penicillin G who received dimethoxyphenylpenicillin and had immediate allergic reactions.

DR. ROMANSKY: Rather large doses of this new agent are required and although economics are not the essential thing in the presence of an acutely ill patient, one wonders what the cost per day would be for this material. Dr. Allen, do you have any figures on the cost per gram.

DR. ALLEN: I think we'll have to refer this question to Bristol.

DR. ROMANSKY: When other agents were used, for example, chloramphenicol and erythromycin, we notice for some peculiar reason that clinicians are hesitant to go above a certain dose. Yet we know patients have received 50 and 100 million units of penicillin intravenously, or 30 to 60 g of penicillin G a day, and yet there is a reluctance to use doses of tetracycline, erythromycin, above 2 g a day. We have used 6 and 8 g a day and certainly in this type of infection it's completely justified to use large doses of these other agents. I do not say they should be used in conditions where organisms are resistant. The clinicians are reluctant for some reason or other to get above what is classified as the so-called standard daily dose.

DEMETHYLCHLORTETRACYCLINE IN PEDIATRICS

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INTRODUCTION

Demethylchlortetracycline (DMCT), first described by McCormick et al. [1] in 1957 has been the subject of extensive clinical pharmacologic studies [2-8]. The absorption of this drug after oral administration has been determined. Its excretion in urine and bile, plasma protein bindings, and renal clearance has been measured and compared with previously known tetracycline compounds.

Antibacterial effect of demethylchlortetracycline has been shown to be greater than other tetracyclines, with a comparable range of activity. Toxicity has been reported as similar to that of tetracycline with slightly higher incidence of nausea, vomiting, and diarrhea on comparable doses. Because of its greater activity and greater resistance to degradation by acid and alkali, the serum activity is greater and more prolonged. As a consequence of this lower doses can be used, thereby minimizing unpleasant reactions.

In spite of the vast number of investigations carried out on DMCT, relatively few studies have been done in pediatrics. This study was designed to determine optimum dosage, serum levels, toxicity, antibacterial range of activity, and clinical effectiveness of demethylchlortetracycline in the pediatric age group.

MATERIALS AND METHODS

Antibiotic. Demethylchlortetracycline was supplied as drops containing 60 mg/ml; syrup containing 75 mg/5 ml; and capsules of 75 mg and 150 mg.*

Assays of antibiotic levels in serum. Four, 8, and 12 hr after administration of a single 5 mg/kg dose of DMCT syrup, blood was collected by venipuncture. The blood was allowed to clot, and was centrifuged. The serum was pipetted off and stored at a -5 C. When an adequate number of specimens were collected, they were coded and shipped in dry ice to Lederle Laboratories where serum concentrations and tetracycline equivalents were determined.

Antibacterial spectrum. Sensitivity studies were done on 602 bacterial cultures from significant clinical sources using antibiotic impregnated disks purchased from Baltimore Biological Laboratories. These discs contained the following approximate amounts of antibiotic: penicillin, 2 units; tetracycline, 5 μg; DMCT, 5 μg; erythromycin, 2 μg; oleandomycin, 2 μg; chloramphenicol, 5 μg; novobiocin, 5 μg; streptomycin, 2 μg; furadantin, 100 μg; neomycin, 5 μg; and polymyxin, 50 μg. Interpretation of sensitivity was read strictly according to the presence or absence of a zone of inhibition.

Clinical studies. DMCT was given to patients with presumed bacterial infections seen in the outpatient department of the Permanente Medical Group, Fontana.

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*Kindly supplied as Declomycin by Dr. Gordon R. Dempsay, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

Table 1. Sensitivity Pattern of 337 Gram-Negative Organisms

Organism	Total cultures	DMCT		Tetra-cycline		Chloram-phenicol		Penicillin		Erythro-mycin		Oleando-mycin		Novo-biocin		Baci-tracin	
		S*	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
Escherichia coli	170	111	59	120	50	162	8	132	38	140	30	135	35	150	20	118	3
Aerobacter	60	28	32	26	34	44	16	33	27	41	19	32	28	45	15	26	2
Pseudomonas	54	3	51	4	50	14	40	31	23	33	21	49	5	2	52	39	15
Proteus	40	—	40	—	40	23	17	21	19	18	22	6	34	20	20	16	6
Alcaligenes	13	5	8	5	8	9	4	10	3	11	3	11	2	3	10	11	2

*S = sensitive
R = resistant

Table 2. Sensitivity Pattern of 265 Gram-Positive Organisms

Organism	Total cultures	DMCT		Tetra-cycline		Chloram-phenicol		Penicillin		Erythro-mycin		Oleando-mycin		Novo-biocin		Baci-tracin	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
Staphylococcus aureus	225	154	71	156	69	209	16	100	125	208	17	206	19	212	13	211	14
Micrococcus epidermidis	40	23	17	24	16	39	1	20	20	38	2	35	5	38	2	28	12

All patients were evaluated one week later to determine response and toxic side reactions. The drug was given to 38 patients admitted to Los Angeles Children's Hospital who were observed daily.

RESULTS

Serum activity was determined by the twofold dilution method using *Bacillus cereus* as the test organism and was expressed in tetracycline equivalents. Levels were obtained that ranged from 2-10 μg in 4 hr and fell to between 0.6-1.2 μg in 12 hr. Three simultaneous cerebrospinal fluid level determinations showed failure of the antibiotic to cross the meninges in concentrations higher than 0.3 μg/ml.

Sensitivity studies done on 602 organisms submitted to the laboratory from the outpatient department revealed that the sensitivity pattern on all organisms tested was essentially the same for both tetracycline and DMCT. Table 1 outlines the pattern for Gram-negative organisms and Table 2 for Gram-positive organisms.

CLINICAL EVALUATION

DMCT was given for 5-14 days to 150 outpatients with a variety of common bacterial infections, the majority of which were otitis media and tonsillitis. As seen in Table 3 only 7 patients out of 68 with otitis media failed to improve. No significant pathogens were cultured from the ears of those patients failing to respond. Surgical management was necessary to effect a cure in most instances where patient failed to respond to DMCT.

Table 3. Clinical Effectiveness of Demethylchlortetracycline

Disease	Total patients		Cured		Improved		Not improved	
	Out	In	Out	In	Out	In	Out	In
Otitis media	68	5	57	5	4		7	
Tonsillitis	40	4	40	4				
Bronchitis	13	3	13	3				
Lymphadenitis	15		14		1			
Pneumonia	4	9	4	8				1*
Cellulitis	2	6	2	6				
Sinusitis	5	1	5	1				
Pertussis	2		2					
Pyelonephritis		7		6				1*
Laryngotracheitis		3		3				

*Expired of (1) sarcoma and (2) severe hydronephrosis.

The group of 38 inpatients treated responded well, generally. Two expired, probably becuse of their primary diseases, namely, massive hydronephrosis and sarcoma with pneumonia.

Table 4 summarizes the results of treatment and the incidence of toxic side effects. The inpatient listed with sun sensitivity developed this after discharge from the hospital, and it disappeared 2-3 days after discontinuing the antibiotic.

Table 4. Demethylchlortetracycline Effectiveness and Toxicity

Cases	Outpatient	Inpatient	Total
Cured	138	34	172
Improved	5	0	5
Unimproved	7	2	9
Expired	0	2	2
Total	150	38	188
Sun sensitivity	2	1	3
Nausea and vomiting	3	1	4
Diarrhea	0	3	3

SUMMARY

1. Demethylchlortetracycline is well tolerated in the pediatric age group when given in daily dosgages of 10-15 mg/kg body weight. This results in an adequate serum level when dose is divided into three aliquots and given every 8 hr.

2. Bacteriologic sensitivity studies show a pattern comparable to tetracycline.

3. DMCT administration results in a good clinical response in most common bacterial infections.

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DISCUSSION

DR. KIRBY: I want to congratulate the author on his very fine presentation showing that this tetracycline antibiotic is effective in therapy. There are a couple of points I would like to raise, however, especially the point in regard to the blood levels. There are many who question whether the concept of tetracycline equivalents has any validity and whether this is in any sense a proper way to measure the blood levels of Declomycin. Specifically, many feel that a serum dilution method measuring directly activity against a pathogen may make more sense. In doing this type of test with Declomycin, it's surprising how little of this is done. Practically all of the blood levels have been the so-called tetracycline equivalents in terms of antistreptococcal activity. In our experience, at least, it is simply not true that far less drug gives greater activity. In a group of 15 volunteers receiving a single dose of 500 mg of tetracycline, the antistreptococcal activity is considerably higher than that with 300 mg of Decolomycin. This occurs not only after the first dose; we have extended these studies to up to five days. It is true that the Declomycin is more active. It is true that its action is more prolonged. But even after five days, measuring antistreptococcal activity, the lower dose of Declomycin is not greater than the activity with tetracycline and furthermore, in the same studies, 24 hours after discontinuing therapy, there was no evidence of any streptococcal activity with declomycin in contrast to tetracycline.

DR. RUTENBERG (Boston): There have been quite a number of published reports on both the efficacy and the lack of toxicity of Declomycin. On the other hand, there have been some reports, such as Dr. Kirby just described, which deal with comparable or the lack of increased efficacy of Declomycin compared to other tetracyclines. Also, there have been some rumblings about the increased toxicity of Declomycin as compared to the other tetracyclines. These toxic reactions have dealt with photosensitivity reactions in the skin which simulate second degree burns. There is such a report from Lind and Trafton from Boston. There have been indications of possible liver toxicity such as impairment of liver function after two or three weeks of Declomycin therapy and the usual type of gastrointestinal reactions with Declomycin the same type that occur with chlortetracycline, with oxytetracycline, with tetracycline, particularly if Declomycin is given in the same dosage as the other tetracyclines. From what Dr. Kirby said, apparently this is one of the requirments. I would like to hear a little more from the various investigators who are here, possibly from the speaker, as to whether or not some of the reactions which I described are of significance in the light of this questionable superiority of Declomycin against the microorganisms.

DR. ALLEN: Our experience on the toxic reactions was that they were very minor in nature. The patients who exhibited vomiting required cessation of the drug. The ones with diarrhea continued on medication in spite of the diarrhea with no apparent severe difficulty. The sun sensitivity reaction that was reported was a curious thing. Patients all exhibited this after they had been on the drug for 2 or 3 days and when they were exposed to more than the usual amount of sun they became quite flushed and complained of tenderness of the skin. This stopped within 2 or 3 days after the drug was discontinued or when the patient was removed from exposure to the sunlight. This first one that I saw this in was my own son who exhibited this after a period of 2 days on treatment. When he went out to the beach he became severely sunburned, not so much so that he actually blistered, but he complained very markedly of this and it was the first thing that brought it to our attention.

DR. KIRBY: Our own experience has been that, and we have been treating only adults, with the Declomycin 150 mg capsule the toxicity is minimal. However, on the basis of our own blood level studies, we do not consider this an adequate dose. Severely ill patients being treated with tetracycline receive 0.5 g four times a day, or 2 g/day. For Declomycin a comparable dose would be at least 0.3 g, four times a day, or a total of 1.2 g/day. With this dosage, the Declomycin toxicity is considerably greater than that with tetracycline.

DR. RIGGIO (Charles Pfizer & Co.): Not to add more confusion to the sceen, Dr. Kirby mentioned one way of evaluating a drug's activity, which certainly sounds quite plausible, the activity dilution. My one coment is about determining the actual weight of the drug in the serum of the patients, that is the tetracycline equivalents. It would seem to me that if you are going to measure the concentration of antibiotic in serum, this should be expressed in terms of a standard of Declomycin and not in terms of a tetracycline standard. This can readily be exemplified if you determine the activity of the two antibiotics against Bacillus cereus. Declomycin is more active against B. cereus. Therefore, when your standard is tetracycline, which is less active, the weight concentration is only an apparent figure approaching 5 μg, whereas if you had taken Declomycin and a Declomycin standard, the weight concentration would come down. This is a minor point. I also wanted to add a theory about photosensitivity reactions. It has been expressed by many investigators that Declomycin has produced this sort of toxic effect, and my investigation in this matter is that all the tetracyclines, that have basic tetracycline arrangements are compounds which have the ability to absorb UV light. They all show a peak at 2500-2900 A which is the range which causes sunburn in human beings. One possible reason why Declomycin will cause a phototoxic reaction, and possibly that the other tetracyclines do not, is that Declomycin stays around longer in the tissue or in the serum than these other tetracyclines, which are either inactivated by serum or excreted more rapidly. Mechanisms of action of sunburn are theoretical and one theory concerns the conversion of histidine to histamine which increases capillary permeability and, thus, reddening of the skin. Another theory is that the amino acids tryptophane, phenylalanine, and tyrosine, which are basic components of dermal protein, also have the ability to absorb UV light. They, in turn, when exposed to sun or 2500 A, are coagulated, thus releasing histamine-like substances or an H-substance, which in turn will increase capillary permeability. If you take a drug which stays in the circulation longer than another drug, and has a serum binding effect to such proteins, you might thus have an enhancement of this natural reaction by having a drug which will absorb UV light and at the same time be in proximity to proteins which absorb UV light. If this were the mechanism, one could see that on excretion of the drug or by metabolic inactivation of it, the sensitizing phenomenon would be a temporary thing and would be abolished when the drug is no longer measurable in the serum.

DR. ROMANSKY: Our experience, although not in large numbers, is quite similar to Dr. Kirby's with Declomycin. The type of patients we have at the City Hospital here are not going to be adequately treated by the smaller doses of Declomycin and the larger doses are required. We also have the same difficulty with increased side reactions.

DEMETHYLCHLORTETRACYCLINE (DMCT) IN THE TREATMENT OF CHRONIC AMOEBIC COLITIS

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INTRODUCTION

Demethylchlortetracycline* (DMCT), a demethylated homologue of the tetracyclines, is a new antibiotic produced by a mutant of Streptomyces aureofaciens Duggar. DMCT differs from chlortetracycline in that it does not have the methyl group at the 6-position of the tetracycline nucleus, is notably resistant to degradation by acid and alkali, and is easily absorbed from the gastrointestinal tract. DMCT, like the other tetracyclines, has a broad spectrum of antibacterial activity. Also, it has lower minimum bacterial inhibitory concentrations, and because of a slower rate of renal clearance, therapeutic levels in the blood and tissues can be maintained for longer periods after relatively small single doses.

This study was undertaken to determine if DMCT might be useful as an antiamoebic agent. This was considered in view of the antibacterial activity and extended blood and tissue levels of DMCT. It is known that the bacterial flora has an intimate environmental influence on Endamoeba histolytica and the colonic lesions which it causes.

It should be noted at this point, that the results obtained with antiamoebic agents can be widely divergent, not only with different but even with the same amoebicides and the same investigators at different times. These apparent paradoxes can generally be ascribed to different criteria being used to decide "cure" or failure of treatment, to different dosages of the antiamoebic agent, to differences in races or strains of E. histolytica and bacterial flora, as well as to failure to distinguish between acute and chronic symptomatic intestinal amoebiasis in published reports.

CLINICAL MATERIAL

In this paper, we present the results of treatment with DMCT of 74 adult patients with chronic amoebic colitis. Sixty-six of these patients had symptoms that were ascribed to infection with E. histolytica.

METHODS

A definitive diagnosis of infection with E. histolytica was made by finding cysts or trophozoites in saline smears or cysts in SAEX concentrations of the feces [6]. The effects of treatment with DMCT were evaluated by similarly examining the feces for the presence of E. histolytica on the fifth day and last day of treatment, and then at weekly intervals during a posttreatment period lasting six weeks. This elective period of six weeks was adopted in previous studies, as well as in this one, so as to avoid the possibility of mistaking reinfection for relapse if E. histolytica should reappear in the feces after that time, particularly in a locality where reinfection was highly probable.

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*The trade name of Lederle Laboratories for demethylchlortetracycline is Declomycin.

Dosage of demethylchlortetracycline (DMCT). Early in the study, a discretionary dosage of 900 mg (six capsules) of DMCT was given daily for 10 consecutive days. After the treatment of the first 15 patients with the 10-day dosage, therapeutic response was evaluated and only one parasitic relapse was encountered. Accordingly it was thought that the dosage could be reduced to 7 days. However, before this change in dosage could be effected, an additional 11 patients were treated with the earlier dosage. Thus, 26 patients were treated with 900 mg/day of DMCT for 10 days and 48 patients received 900 mg/day for 7 days.

Therapeutic results. In all of the cases, E. histolytica disappeared from the feces by the fifth day of treatment. Two of the 26 patients treated with the 900-mg dose administered daily for 10 consecutive days had parasitic relapses at six weeks posttreatment. Of the 48 patients treated with 900 mg daily for 7 consecutive days, four had parasitic relapses, each at six weeks posttreatment. There was no symptomatic or clinical relapse in any of these cases.

In 60 of the 66 cases, in which clinical manifestations were thought to be caused by infection with E. histolytica, these symptoms disappeared promptly with treatment. In the remaining six cases, although partial relief was afforded by treatment with DMCT, it was considered quite possible that there had already occurred permanent bowel damage caused by long-standing amoebic infection and that this was responsible for persisting symptoms.

Toxic reactions. Except for mild to moderate anorexia during treatment with these dosages of DMCT, we observed no other adverse reactions. There were no instances of hepatic, nephric, hemic, or dermal reactions. These dosages of DMCT caused no immediate or delayed allergic reactions.

SUMMARY AND DISCUSSION

Seventy-four adult patients with chronic amoebic colitis were treated with DMCT. Twenty-six of these patients were treated with daily doses of 900 mg for 10 consecutive days, and 48 patients were treated with 900 mg doses administered daily for 7 consecutive days.

These two dosages of DMCT provided essentially similar therapeutic results: 24 of 26 patients successfully treated, and 44 of 48 patients successfully treated.

In view of the consistent disappearance of E. histolytica from the feces in all cases by the fifth day of treatment, it would seem that 900 mg of DMCT administered daily for 7 consecutive days is adequate for treatment of the average case of chronic amoebic colitis.

Also, in view of these highly favorable results, that is 91% successful treatments as evaluated during a six-week posttreatment period and disappearance of E. histolytica from the feces by the fifth day of treatment, it might be possible to reduce the dosage still further to 5 consecutive days of therapy, or to change the schedule of dosage to 4 consecutive days of therapy which would be repeated five weeks later.

It was appreciated that less satisfactory results were obtained by Elsdon-Dew et al. in the treatment of amoebiasis in Durban, South Africa. They ascribed the relative ineffectiveness of DMCT (8 failures in 12 cases treated with 600 mg daily for ten days) to rapid absorption and high blood level. Although we agree with their view that combined therapy of amoebiasis is more effective than treatment with a single amoebicide, we believe that the potential effectiveness of an amoebicide depends not only on luminal or fecal concentrations but is also directly related to concentrations attained in the bowel wall tissues. Another significant difference in their 12 cases was that these investigators gave 600 mg of DMCT daily.

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THE INTRAOCULAR PENETRATION OF DEMETHYLCHLORTETRACYCLINE*

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INTRODUCTION

In 1957 a demethylated derivative of the tetracycline group was described by McCormick, isolated from a mutant strain (S-604) of Streptomyces aureofaciens Duggar (A-377 soil isolate) [1]. It is characterized by its resistance to alkalies and acids and has clinically proved its efficacy in a wide variety of systemic infections normally susceptible to the tetracyclines. The antibiotic has been shown to have a half-life in the blood up to 44% longer than tetracycline. This has allowed dosage rates of 60% that of its tetracycline predecessors and a lowered incidence of toxic and side reactions.

The present study was undertaken to determine the ocular penetration of demethylchlortetracycline (hereafter referred to as DMCT) under normal and certain abnormal ocular conditions. The routes of administration were intraveneous single doses, topical instillations of buffered ophthalmic solutions, and subconjunctival injections. Observations of tolerance and toxicity were made, but were not quantitatively investigated.

MATERIALS AND METHODS

Ninety-two pigmented rabbits were used in the experiments. Prior to ocular manipulations the eye was copiously irrigated with sterile saline and sterile tetracaine (0.5%) was instilled into the lower cul-de-sac. Aqueous and vitreous humor samples were aspirated by use of sterile tuberculin syringes wetted with 1% heparin, using 27- and 18-gauge needles respectively. The rabbits were sacrificed by an intravenous overdose of sodium pentothal prior to vitreous aspirations. All intravenous injections were made in the lateral marginal vein of one ear and the blood samples were removed from the opposite ear. The blood was centrifuged and the plasma used for bioassay.

The technique employed for the assay of DMCT in test fluids was the twofold serial tube-dilution method. Brain-heart infusion broth (Difco), $\frac{1}{2}$ ml, was used in standard culture tubes. Since the aqueous humor was obtainable only in 0.25 ml quantities, the humor of four eyes from identical test groups was pooled for each sample. The 0.5-ml volumes of the assay material were diluted 1:2 with double strength broth. Twofold dilutions were then made by serial passage of 0.5 ml of the preceding dilution into 0.5 ml of broth, and carried from 1:2 to 1:512. A sensitive strain of Bacillus subtilis, ATCC 6633, was employed in the bio-assay procedure. An overnight trypticase soy agar slant subculture of the organism was scraped and suspended in sterile normal saline to give an OD of about 65-75 units on the Klett-Summerson photoelectric colorimeter, using a number 54 green filter and normal saline as a blank. This bacterial suspension was added in 0.025-ml amounts as the inoculum for each tube. The assays of ocular fluids and plasma were read after 24 hr incubation at 30 C. By the pour-plate technique, 0.025 ml of a Klett 70-unit suspension of bacteria was found to contain roughly 5000 viable organisms.

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*Supplied by Lederle Laboratories, Pearl River, N.Y. (Declomycin, Lederle).

The DMCT test materials for penetration study were freshly prepared from the powdered drug under aseptic conditions. Designed specifically for intravenous use, it consisted of 375 mg of DMCT as the hydrochloride and 150 mg of ascorbic acid. The pH of a solution containing 10 mg/ml was 2.4. Vials of prepared 0.5% ophthalmic powder and diluent (distilled water) were stated to contain DMCT as the hydrochloride, 0.02593 g; sodium borate, 0.01500 g; and sodium chloride, 0.06250 g; and, mixed into solution for topical instillation, the pH of this was 7.9. For standardization of inhibiting concentrations, the DMCT was serially diluted both arithmetically and geometrically. In six such procedures, after 24 hr, 0.025 to 0.03 μg/ml of drug completely and consistently inhibited the growth of 0.025 ml of B. subtilis suspension. By this method the minimum drug present in the first tube (1:2 dilution) of a sample series must be at least 0.03 μg/ml. Less than 0.06 μg/ml would therefore not be detectable in the sample being tested. Controls were run concurrently to guard against contamination.

An experimental intraocular inflammation was produced by the firm contact of a Hyfrecator needle placed 2-3 mm from the limbus in a 90 degree arc around the eye. The contact, lasting for 7-10 sec caused immediate local conjunctival and ciliary vessel congestion, and the underlying area became too hot to be touched. This insult was transient, and rabbits showed little sign of inflammation after 4 days. Tetracaine was instilled prior to the injury, and the rabbits did not become agitated during the procedure.

The corneal abrasions were made under local anesthesia by scraping the entire cornea free of epithelium with the flat edge of a dull keratome.

REACTIONS

Systemic. Toxicity studies with single intravenous doses up to 50 mg/kg produced no signs of systemic or ocular reaction or injury. It was noted that intravenous injections consistently produced evidence of pain in the rabbits, and was aggravated when their struggling resulted in extravascular drug deposition.

Topical. Preparations of 0.5% aqueous DMCT solution in the form of eye drops were given at a rate of two drops each 5 min for 20 min. There was no apparent reaction of the external ocular tissues. Similar applications with 1% solution resulted in a marked hyperemia, minimal chemosis of the palpebral conjunctiva, and increased sensitivity of the rabbits to eye manipulations. There were no signs of corneal damage to gross inspection. This reaction subsided almost completely in 24 hr. Administration of 0.5% drops to the completely abraded cornea produced little more irritation than that seen in the unmedicated abraded corneas which served as controls.

Subconjunctival. The DMCT powder used for these injections was stated by the manufacturers to be suitable only for intravenous injection and, similar to its analogues, to be irritating to the subcutaneous and muscular tissues. All doses were given in 0.2 ml volumes. As detailed below, large subconjunctival doses resulted in a substantial number of prolapses of choroid and vitreous humor accompanied by hemorrhage through the sclera at the site of injection. This occurred while the eye was proptosed for aqueous humor extraction. It did not occur with the 2-mg dose but was seen in 3 of 20 eyes with the 4-mg dose and in 6 of

10 eyes following the 20-mg dose. The 2-mg injections produced only a transient hyperemia and mild chemosis which resolved almost completely in 48 hr. There were no corneal effects with this dosage level.

RESULTS

Systemic. An initial series of single 2-mg/kg DMCT intravenous injections showed no evidence of intraocular drug, so the bulk of studies were conducted with a standard dosage of 4 mg/kg. This is equivalent to an initial dose of 300 mg of DMCT in the 70-kg human. 4-mg/kg administrations yielded no detectable aqueous drug levels in the normal or in the inflamed eye (Table 1). However, a prolonged and stable plasma level maximally noted at 1.9 μg/ml was well maintained through the 7 hr testing period. Single injections of 10 mg/kg tested in 3 and 6 hr produced plasma levels up to 7.7 μg/ml with 3 hr maximal aqueous drug levels of 0.48 μg/ml both in the normal and in the inflamed eye. Fifteen and 20 mg injections yielded slightly higher plasma and aqueous drug levels in 3-hr tests.

Table 1. Intraocular Penetration of DMCT Administered Intravenously

Dose of administration	DMCT levels, μg/ml		
	Hours*	Plasma	Aqueous†
Normal eyes			
2 mg/kg	2	1.9	<0.06
	3	0.24	<0.06
4 mg/kg	1.5	0.48	<0.06
	2.5	2(0.48) 0.96	2(<0.06) 0.12
	3.5	0.48	<0.06
	4.5	1.9 0.96	2(<0.06)
	7	0.48	<0.06
10 mg/kg	3	3.8	0.24
20 mg/kg	3	3.8	0.24
Inflamed eyes			
4 mg/kg	1.5	2(0.96)	2(<0.06)
	3	0.48	<0.06
	6	0.48 0.24	2(<0.06)
10 mg/kg	3	7.7	0.48
15 mg/kg	3	3.8	0.12
	6	1.9	<0.06

*Hours represent time elapsed from DMCT administration to removal of sample.

†Each figure represents pooled aqueous humor from 4 eyes.

Topical. Application of 0.5% buffered DMCT solution in normal or in inflamed eyes failed in all cases to cause aqueous penetration in detectable amounts after 1 hr (Table 2). Administrations into the abraded eye yielded aqueous drug levels on the order of 0.96-1.9 μg/ml in 1 hr. One percent eye drops in the normal eye produced DMCT aqueous values of 0.24 and 0.48 μg/ml after $^1/_2$ hr. No DMCT vitreous levels were detected in the normal, abraded, or inflamed eye in 1 hr.

Subconjunctival. All injections of DMCT into the normal eye produced measurable aqueous humor drug concentrations (Table 3). The unbuffered drug in a 20-mg dose (pH 1.6) produced aqueous drug concentrations in two instances greater than 30 μg/ml. This was attributed to the extremely irritating effect of this injection, with vitreous human prolapse as detailed above. Four-milligram

Table 2. Intraocular Penetration of DMCT Administered Topically

Concentration of application	DMCT levels in μg/ml		
	Hours*	Aqueous†	Vitreous†
1% solution			
Normal eyes	0.5	0.48 0.24	
0.5% solution			
Normal eyes	1	2(< 0.06)	2(< 0.06)
Inflamed eyes	1	3(< 0.06)	3(< 0.06)
Abraded eyes	1	1.9 0.96	2(< 0.06)

*Hours represent time elapsed from DMCT administration to removal of sample.
†Each figure represents pooled aqueous or vitreous humor from 4 eyes.

Table 3. Intraocular Penetration of DMCT Administered Subconjunctivally

Dose of administration	DMCT levels in μg/ml		
	Hours*	Aqueous†	Comment
Normal eyes			
2 mg/0.2 cc	1	0.24 0.12	
4 mg/0.2 cc	1	4(0.48) 0.24	Prolapse of 3/20 eyes
20 mg/0.2 cc	1	2(> 30.)	Prolapse of 6/10 eyes

*Hours represent time elapsed from DMCT administration to removal of sample.
†Each figure represents pooled aqueous humor from 4 eyes.

doses (pH 2.25) yielded drug levels in 1 hr of 0.48 μg/ml, with one of five samples being 0.24 μg/ml. Two-milligram doses (pH 2.4) produced readings of 0.12 and 0.24 μg/ml.

DISCUSSION

Systemic. The results of single intravenous administrations of DMCT in the normal rabbit appear comparable with reports of the other tetracyclines. Injected in doses of 50 mg/kg, oxytetracycline yielded less than 2.5 μg/ml [2]. Another study made 100 min after injection of 30 mg/kg showed an aqueous drug level of 3 μg/ml [3]. Chlortetracycline was stated to produce maximum aqueous drug levels of the order of 0.2 μg/ml, with 0.1 μg/ml in the vitreous following dosages of 20 mg/kg [4]. In another report chlortetracycline did not penetrate the aqueous humor in detectable concentrations after 100 min following injection of 30 mg/kg [3]. Tetracycline in the rabbit is reported to yield 0.3 and 0.2 μg/ml of aqueous drug concentration in 2 and 4 hr, respectively, after 50-mg/kg injections [4]. However, other workers found that the intraveneous injection of over 20 mg/kg caused the death of the rabbit [5]. The same study demonstrated that, in the normal human’ after 11 hr, two 7 mg/kg doses each 6 hr yielded high aqueous DMCT levels ranging from 34 to 98 μg/ml.

Multiple dosage schedules were not investigated in this project, and the human-weight equivalent of 750 mg in a single injection (10 mg/kg) was required to achieve detectable aqueous DMCT levels no higher than 0.5 μg. In this study neither the normal nor the inflamed eye yielded detectable amounts of DMCT after injections of 4 mg/kg. There was no increase in aqueous humor drug concentrations following inflammation of the eyes and high doses.

Topical. Demethylchlortetracycline in the 0.5% solution penetrated the abraded cornea in significant concentrations while failing to be detected in all procedures with the intact cornea. This further confirms the observations of other workers on the effectiveness of the corneal epithelial barrier to drug penetration [4,6]. Severe inflammation of the ciliary body failed to enhance aqueous drug levels. The 1% DMCT solution produced a minimal detectable amount into the aqueous humor, but was regarded as too irritating, although transient, to be of satisfactory use in routine ocular therapy.

Subconjunctival. Chlortetracycline, due in part to its low solubility, has been found to give inadequate aqueous humor drug levels in the doses deemed satisfactorily nonirritating. Oxytetracycline injections of more than 20 mg are poorly tolerated producing marked hyperemia and corneal haziness [2]. In another study of chlortetracycline, subconjunctival injections were irritating to such an extent that the procedure was considered contraindicated [1]. Tetracycline is reported to produce an extreme local reaction in injections over 2.5 mg (5 mg/ml), and in 5 mg doses (10 mg/ml) the abraded eye absorbs six times more drug than does the normal eye (37 vs 6 μg/ml) in 1 hr [4]. In this same study vitreous humor drug values of 3 μg/ml were obtained in the normal eye after 1 hr. Injections of 1 mg of tetracycline in 0.5 ml of solution has produced maximal aqueous drug concentrations to 18 μg/ml in 2 hr, with variability down to 5 μg/ml [5].

The results of DMCT penetration into the normal eye suggest the value of further studies with abnormal eyes, since concentrations of 0.24 and 0.48 μg may be of borderline value in the management of infections due to susceptible organisms [7,8]. Studies with buffered subconjunctival solutions (pH 7.2-7.9) are in progress. Preliminary work indicates that a part of the local reaction is eliminated and that drug levels are obtained comparable to those observed in the aqueous humor. If these impressions can be confirmed, the establishment of higher margins of safety and increased drug tolerance may justify trials of DMCT in the subconjunctival treatment of selected intraocular infections.

SUMMARY

1. On the basis of published studies demethylchlortetracycline (DMCT) in comparable intraveneous single doses yields slightly higher aqueous humor drug levels than chlortetracycline and tetracycline and slightly lower levels than those reported for oxytetracycline. DMCT aqueous humor levels after high doses suggest limited value of this drug in the treatment of intraocular infections.

2. Single intravenous doses equivalent to a single 300-mg dose in the adult human yield less than 0.06 μg/ml in either the normal or the inflamed eye.

3. The study demonstrated persistent plasma drug levels during the period of examination (up to 7 hr).

4. Topical application of 0.5% DMCT solution is of little value in intraocular disease not involving the cornea. It may, however, be recommended for trial in selected anterior segment infectious disease in which the continuity of the corneal epithelium has been interrupted.

5. One percent solutions penetrate the normal eye, but are felt to be too irritating for clinical use.

6. The amount of subconjunctival penetration of DMCT is significant, but the material now available does not offer an adequate safety margin against marked irritation, and on this basis is not recommended for clinical use. A properly buffered solution may be a partial answer to this problem.

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DISCUSSION

DR. SEELIGER (University of Bonn): You have given the results of pooled samples, as I understood you. Did you notice any differences when you did tests on the single specimens? We have done a few preliminary studies with Professor H. Leidhecker, from the Eye Clinic in Bonn, and have found, so far, very low levels, but with quite some difference when we tested various eyes. It means in one eye the level was considerably higher than the other. Was that the reason that you used pooled specimens?

A: No, sir, pooled specimens were used because our type of method demanded it. The 1/4 ml of aqueous humor that you can get from one eye was inadequate for our method and we used it as a matter of convenience more than a matter of desire.

DR. SEELIGER: We used the plate-diffusion method.

DR. FURGUIELE: We used the pooled aqueous because in the tube-dilution method that we employed we needed a volume of about 1/2 cc to make our work come out consistently. We did it with a single eye with previous antibiotics and it didn't work out as well for serial dilution method.

INTRAPERITONEAL KANAMYCIN IN THE TREATMENT OF PERITONITIS*

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INTRODUCTION

The intraperitoneal instillation of a safe and effective antibiotic would be a valuable adjunct in the treatment of peritonitis. Many antibacterial agents found to give good therapeutic results when administered by other routes have been found unsuitable for intraperitoneal administration. Recent experimental studies have shown that kanamycin can be given intraperitoneally in large doses without local or systemic toxic effects [1]. In this regard kanamycin is superior to other antibiotics currently available for intraperitoneal administration.

The present study reports the effect of intraperitoneal kanamycin on experimental peritonitis in dogs and summarizes preliminary data obtained from the intraperitoneal administration of kanamycin to 75 patients treated in the Louisiana State University Surgical Service at Charity Hospital in New Orleans.

EXPERIMENTAL STUDY

Fifty-seven mongrel dogs weighing from 7 to 25 kg were used. For at least two weeks prior to operation all animals were fed a diet known to be free of antibiotics. Operations were performed aseptically with the dog under Nembutal anesthesia and through a right rectus lower abdominal incision. The animals were offered only water on the night prior to operation, and food and water were withheld for 24 hr postoperatively. Dogs were autopsied as soon after death as possible. Survivors were sacrificed from 10 days to 2 weeks following operation.

Peritonitis was produced in 57 dogs by the following method. The cecum was isolated by dividing its peritoneal attachments and blood supply. The base of the cecum was then tightly ligated with umbilical tape. Following closure of the abdomen, 5 ml/kg of castor oil were given via intragastric tube to each dog.

Control group. Nineteen of 28 untreated dogs died (68%), twelve in 27-55 hr and 7 in 3-8 days. Nine untreated animals (32%) survived.

Treated group. Twenty-nine dogs received 3 g of kanamycin powder intraperitoneally following ligation of the cecum. One gram of powder was sprinkled into each lower quadrant and 1 g into the midportion of the abdominal cavity. No other therapy was given these animals. Twenty-one (72%) of the animals survived. Six dogs died between 4 and 8 days and the remaining two on day 11 and 12 respectively (Table 1).

Autopsy findings. The untreated dogs that died between 27 and 55 hr had from 50 to 250 ml of a foul sanguino-purulent fluid in the peritoneal cavity. The appendix was usually necrotic beyond identification and there was a severe generalized peritonitis. The tape ligature was loosely attached at the base of the cecum and gross perforation of the bowel in this area was usually evident. Those animals

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*This work was aided by research grant E-1600 from the National Institute of Allergy and Infectious Diseases, Public Health Service, and by a grant from Bristol Laboratories.

Table 1. Time of Death in Dogs with Experimental Peritonitis

Group	Number of deaths			
	27-55 hr	3-8 days	11-12 days	Total
Control, 28 dogs	12	7	0	19
Treated, 29 dogs	0	6	2	8

that died between 3 and 8 days had up to 350 ml of pus in the abdominal cavity, generalized peritonitis, and, in 3 of the 5 cases, a periappendiceal abscess was present.

The eight treated dogs that died had autopsy findings similar to those control animals that expired between 3 and 8 days. The amount of purulent peritoneal fluid in the treated dogs, however, ranged from 100 to 800 ml, significantly more than that found in the control group.

Postmortem findings were essentially the same in survivors of both treated and control groups sacrificed at 2-5 weeks. Approximately half of the animals had small, localized periappendiceal abscesses with dense adhesions in this area. The remainder had only periappendiceal adhesions.

Laboratory data. Hematocrit, total white blood cell count, and differential white cell count were done preoperatively and 24 hr following operation on 18 control and 14 treated dogs. There were no differences between treated and untreated survivors. Among those animals that died, however, the rise in total white blood cell count was higher in those that received intraperitoneal kanamycin than in those of the control group. Changes in hematocrit were not remarkable.

CLINICAL STUDY

Seventy-five surgical patients operated upon at Charity Hospital received intraperitoneal kanamycin in conjunction with surgical procedures. The age group ranged from 7 days to 77 years and the ratio of males to females was 2:1.

Approximately 80% of the cases were surgical emergencies. Prior to closure of the peritoneum, 1 g of kanamycin powder was dissolved in 50 ml normal saline and instilled into the peritoneal cavity. Occasionally, 2 g of the drug were used with 100 ml saline. All patients in this study also received parenteral antibiotics. Penicillin, streptomycin, and chloromycetin were used most often.

The types of cases are listed in Table 2. Although in the majority of patients there was gross infection or contamination of the peritoneal cavity, intraperitoneal kanamycin was used occasionally prophylactically with procedures such as decompression enterostomy during operation for simple intestinal obstruction.

The most common indication for intraperitoneal kanamycin therapy in this series was acute perforated appendicitis. Either generalized peritonitis (present in 19) or periappendiceal abscesses (in 13) were noted in all 29 of these cases. Kanamycin was not used in acute appendicitis without perforation. The organisms cultured from peritoneal fluid obtained at operation in 24 cases are listed in Table 3.

Aerobic and anaerobic culture methods were used and, although routine sensitivity studies at this hospital do not include kanamycin, in a few instances sensitivity tests of the organisms to kanamycin were obtained by special request. In these cases kanamycin was found to be effective against the Pseudomonas, Escherichia, Aerobacter, and paracolon organisms.

Table 2. Diagnoses in 75 Patients Receiving Intraperitoneal Kanamycin at Operation

Diagnosis	Number of cases
Gastrointestinal perforation	
Acute perforated appendicitis	29
Perforated peptic ulcer	9
Traumatic perforation of stomach	3
Perforation of colon	5
Perforation of small intestine	3
Intestinal obstruction, gangrene	5
Intra-abdominal abscess, nonappendiceal	
Leaking duodenal stump	2
Leaking gastrostomy site	1
Liver abscess	4
Elective colon surgery with abnormal contamination	5
Miscellaneous	9
Total	75

Table 3. Human Cases of Perforated Appendicitis. Cultures at Operation.

Organism	Number of cases
Escherichia coli	15
Aerobacter aerogenes	6
Pseudomonas aeruginosa	3
Paracolon	3
Enterococci	2
Aerobic Gram-positive rods	4
Gram-positive cocci; diphtheroids; α-hemolytic streptococci	1 each

Toxic effects noted with parenteral kanamycin were not seen in this study The respiratory depression, which may accompany the intraperitoneal administration of neomycin, did not occur with kanamycin.

Postoperative complications (Table 4) included 17 wound infections, 13 of which occurred in patients with generalized peritonitis secondary to perforated appendicitis. All wound infections responded to incision and drainage and subsequently healed without the necessity of secondary closure.

Patients with severe peritonitis had a smoother postoperative course than would be ordinarily expected. Patients with peritoneal contamination due to gunshot wounds, stab wounds, or elective colon surgery had remarkably few postoperative complications. One patient was a 7-day-old baby with perforation of the splenic flexure of the colon secondary to anal stenosis. Although peritonitis was

Table 4. Postoperative Complications in 75 Patients Receiving Intraperitoneal Kanamycin

No complications	45
Wound infection	17
Cul-de-sac abscess	2
Deaths	11
Deaths attributed to kanamycin	0
Total	75

severe and the peritoneal fluid contained Clostridium perfringens and Clostridium septicum, the patient recovered without postoperative complications. Another patient was a 22-year-old male with an extensive upper abdominal knife wound. Despite complete exposure of the upper peritoneal cavity, incisions in the liver, stomach, and diaphragm, and the presence of large amounts of grass and leaves throughout the peritoneal cavity, he was discharged on the fourteenth hospital day having had a smooth postoperative course.

None of the eleven deaths in the series could be attributed to the use of intraperitoneal kanamycin. The two deaths in the appendicitis group included one patient who entered the hospital in septicemic shock and died 20 hr after operation, and another whose postoperative course was without incident until the sixth day when she developed septicemia due to a urinary tract infection. Three patients with liver abscesses died one week or more following operation. Three deaths occurred in patients who received intraperitoneal kanamycin at reoperation for evisceration or intra-abdominal abscess secondary to previous surgery. Kanamycin was not used at the original operation, and death occured too long after the second procedure for kanamycin to have been an etiologic factor. Two patients died two weeks following palliative surgery for invasive carcinoma of the stomach and benign pyloric obstruction, respectively. The eleventh death occurred 48 hr following resection of gangrenous obstruction of the colon in a 64-year-old man with congestive heart failure.

DISCUSSION

Kanamycin, discovered by Umezawa and associates in 1957 [2], has been accepted as a valuable antibacterial agent. Various reports have indicated the effectiveness of the drug when used topically [3], parenterally [4, 5], and orally for intestinal antisepsis [6].

The wide antibacterial spectrum and low toxicity of kanamycin prompted the investigation of this drug as an intraperitoneal antibiotic. Cohn and Beauclair compared the intraperitoneal administration of tetracycline, neomycin, and kanamycin in anesthetized and unanesthetized rabbits and dogs [1]. They concluded that tetracycline should not be used intraperitoneally because of its irritative effects, neomycin is safe only when the dosage is carefully controlled, and that kanamycin should be the drug of choice when intraperitoneal injection is indicated. They also noted that serum levels following injection of 1 g of kanamycin were comparable to levels achieved by other routes of administration.

Prigot et al., [7] employed intraperitoneal kanamycin in 30 patients with either established peritonitis or peritoneal contamination and found that, "infection was controlled, morbidity was decreased, and all patients recovered without complications."

The experimental and clinical data provided by the present study support kanamycin as the antibiotic of choice for intraperitoneal administration. The significant increase in survival of kanamycin-treated dogs with severe peritonitis is attributed to the high potency of the drug as an antibacterial agent locally as well as systemically after its rapid absorption from the peritoneal cavity.

The design of the clinical portion of this report is such that the absence of toxicity and adverse effects from the use of intraperitoneal kanamycin must be emphasized rather than its therapeutic effectiveness. Without exception, the large house staff involved in the present study attribute improved clinical results to the intraperitoneal administration of kanamycin at operation.

Valuable information regarding toxicity has been obtained from this study. There were no instances of respiratory depression or hypotensive reaction resulting from the administration of kanamycin with the patient under anesthesia. These adverse effects have been reported with the use of neomycin [8] and have led to the introduction of the technique whereby a plastic tube inserted into the peritoneal cavity is used for the injection of neomycin after the patient has recovered from anesthesia. The absence of untoward effects in the 75 patients of the present series eliminates the necessity for this tube when kanamycin is used.

No instances of ototoxicity reported to occur with parenteral kanamycin therapy [9] were noted following the single intraperitoneal injection. Renal toxicity [10, 11] was similarly absent in these patients.

Kanamycin has now been demonstrated to be safe and effective for intraperitoneal use in addition to its value when administered by other routes.

SUMMARY

1. Intraperitoneal kanamycin effectively increased survival in experimental peritonitis in dogs.

2. One gram of kanamycin was administered intraperitoneally at operation to 75 patients with peritonitis or contamination of the peritoneal cavity. There were no examples of hypotension or respiratory depression during anesthesia. Renal and otic toxicity were similarly absent in these patients.

3. Patients with established peritoneal infection or severe contamination of the peritoneum had an unexpectedly smooth postoperative course when intraperitoneal kanamycin was used at operation.

4. These data support the acceptance of kanamycin as the antibiotic of choice for intraperitoneal administration.

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DISCUSSION

Q: It was my impression that toxic effects from neomycin occurred only with large doses of neomycin.

DR. COTLAR: Last year we did some studies comparing neomycin, kanamycin, and tetracycline in rabbits and dogs and we found that the lethal dose of kanamycin in dogs with anesthesia was never reached in these studies, whereas the lethal dose in dogs with anesthesia given neomycin, was somewhat less than 200 mg/kg of body weight. In animals without anesthesia, again the lethal dose in dogs was not determined since it went up well above 2500 mg/kg, whereas in neomycin it was considerably less. One thing that we were impressed with was the narrow margin of safety between the lethal dose and safe dose in experimental animals with neomycin, whereas the margin of safety in kanamycin was much larger. Many surgeons state that if you give a gram or less of neomycin under anesthesia you are not going to have any trouble, but with larger doses, one may produce respiratory arrest—with doses such as 2 g of neomycin intraperitoneally. On the other hand we have given up to 2 and 3 g of kanamycin intraperitoneally to patients without untoward effects.

DR. KAY (New York): Is there any proven advantage of giving kanamycin intraperitoneally over giving it systemically?

DR. COTLAR: This is a good question. I don't know the answer. It seems good sense to me that if you have an adequate local level as well as a systemic level of an antibiotic you would probably get a better response in killing off bacteria. However, I am also impressed with the fact that, since sulfonilamides, investigators have studied just about every chemotherapeutic and every antibiotic agent to study its effect when given intraperitoneally. Such studies have been done using terramycin and other antibiotics. However, in those cases the systemic level was usually good, and occasionally the intraperitoneal administration did increase survival. We have not done it with kanamycin. Shott has shown that despite the almost immediate rise in systemic level with terramycin, there is still a considerable level of antibiotic present in the peritoneal cavity even after 24 hr. Hence it probably is still exercising both local and systemic effects.

DR. JOHNSON (New York): I would like to know why you say systemic as against intraperitoneal. I should think that intraperitoneal is systemic administration. It is absorbed very rapidly into the blood. I think that this is something which must be realized especially by clinicians that when you give a drug intraperitoneally, it is in essence a systemic form of therapy.

DR. COTLAR: Yes, we find that if you give kanamycin intraperitoneally, you get just as good absorption and just as high blood levels as if you had given it by the parenteral route. I do not mean to imply that systemic and intraperitoneal are different, but we get just as good blood levels intraperitoneally. However, as I mentioned before, you do maintain a level in the peritoneal cavity, a separate level from that which develops systemically.

Q: You mentioned that 1 g was used extensively in treatment for dogs. Do you have information on that?

DR. COTLAR: In a copy of the paper which will be published we report a 7-day-old baby who perforated the splenic flexure of its colon due to an anal stenosis. Ordinarily, at least in our hands, this is a fatal disease. We cultured Clostridium septicum as well as a Pseudomonas from the baby's peritoneal cavity and placed 1 g of kanamycin in this infant's peritoneal cavity. The only complication it developed postoperatively was Escherichia coli O111 gastroenteritis which many babies in a hospital do get and it recovered from this. Thus we have been using 1 g for both adults and infants.

PREOPERATIVE BOWEL "STERILIZATION"—A DOUBLE-BLIND STUDY COMPARING KANAMYCIN, NEOMYCIN, AND PLACEBO*

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INTRODUCTION

For many years antibiotics have been used in preparing patients for gastrointestinal surgery. The drugs have been given alone or in combination in order to achieve maximal elimination of the intestinal flora at the time of surgery. This preparation, accompanied by mechanical cleansing of the bowel, is felt by many to be of distinct advantage in reducing postoperative morbidity and mortality [1, 2].

Recently, Tyson and Spaulding [3] have suggested that intestinal "sterilization" should be restricted to situations such as the presence of significant local vascular disease, significant gastrointestinal obstruction, and extensive surgery. They feel that a thorough mechanical cleansing is sufficient in most instances.

In recent years orally administered neomycin, alone or in combination with other drugs, has been considered to be the agent of choice for bowel "sterilization" by a number of workers in the field [1, 2]. Oral kanamycin has been introduced more recently and also shows significant activity against the normal intestinal flora [4, 5].

The present study was undertaken in order to compare bacteriological and clinical results in five groups of patients receiving (in a double-blind fashion) two different dosage regimens of kanamycin and of neomycin and a placebo.

MATERIAL AND METHODS

Clinical material. The patients were selected by the surgical staff on the general surgical service. Any patient who was to have a preoperative regimen for bowel "sterilization" and who could wait 2-3 days was included in the study. Only those patients with significant intestinal obstruction were excluded. However, a few patients were unavailable for the study because they were placed on a known antibiotic by the surgeons.

An envelope system set up from a random numbers table was used to insure randomization. An envelope was drawn for each patient and a coded drug prescribed. A total of 100 patients (20 in each group) was studied. All patients were followed clinically by the surgical staff and by the investigators.

The drug was usually given over a 3-day period. However, a few patients received $1\frac{1}{2}$ to 2 days of therapy. Identical capsules were prepared to contain either 350 mg of neomycin base, 350 mg of kanamycin base, 500 mg kanamycin base, 500 mg of neomycin base, or placebo†. The base equivalent of 500 mg of the sulfate of either drug is 350 mg base.

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*Supported in part, by a grant from Bristol Laboratories, Syracuse, New York.

†Kindly prepared and supplied, with a sealed code, by Bristol Laboratories.

Two capsules of each preparation were given every hour for 4 doses to start the program, then two capsules were given every 6 hr until the midnight prior to surgery. Sixty milliters of castor oil was given with the first dose of drug and again at noon on the day prior to surgery. A low residue diet was given on the first 2 days of therapy and a liquid diet on the third day. All patients were given at least one enema prior to surgery.

Stool specimens. Whenever possible, specimens were collected daily while the patient was receiving therapy. As soon as available, two postoperative specimens were obtained. These were collected in ½ pint waxed cardboard containers and either cultured promptly or placed under refrigeration. Most stools were cultured within 24 hr but a small number were refrigerated for 2 days.

BACTERIOLOGICAL STUDIES

One gram of feces (or one milliliter of liquid stool) was placed in 9 ml of 0.85% sterile saline solution and was then shaken thoroughly with sterile glass beads. Then serial tenfold dilutions were made down to a 10^{-11} dilution. One milliliter of every other dilution was inoculated in SF broth (BBL). These were incubated for 48 hr at 37 C and then smears were Gram-stained.

In addition, 0.5 ml was placed on the surface of a blood agar plate and 1.0 ml incorporated into a pour plate of desoxycholate agar (BBL). The plate media were incubated for 24 hr at 37 C. If no growth occurred, they were held for another 24 hr. Counts were then made and the organisms subcultured and identified.

Tube-dilution sensitivities to kanamycin and neomycin were run on most strains of Staphylococcus aureus isolated, using the technique of Hewitt and Finegold [6].

Cultures of infected wounds, etc. were made in the clinical bacteriology laboratory.

RESULTS

Bacteriological data and clinical follow-up are not yet complete for two of the 100 patients included in this study. Consequently, the code has not been broken; final data on individual drug and dosage programs will be presented in a later paper. However, from the bacteriological data, four of the groups, 58A 220, 58A 221, 58A 235, 58A 236, can be considered as having received kanamycin or neomycin while the fifth, 58A 237 is presumably the placebo. Most of the patients were in the older age group, the percentage of patients operated on who were over 60 years of age being: 58A 220, 72%; 58A 221, 70%; 58A 235, 83%; 58A 236, 42%, and 58A 237, 77%.

By far the most common diagnosis for which surgery was performed in all groups was a malignancy of the large bowel. The differences in number of malignancies in each group were not remarkable. Benign polyps were next in line. Therefore, it would follow that a great majority of the surgery was performed on the large bowel, and in almost every case the bowel was opened or cut across. The number of patients who had colon surgery was virtually identical in all groups.

Although patients with obstruction were to be excluded, in each group there were two or three cases in which the operating surgeon felt there was a low grade obstruction. However, in no case was there sufficient obstruction to significantly impede the fecal stream. There were two patients with colostomies in group 58A 220 and one in each of the other groups except 58A 237.

Thirty-one of the 59 cases in which the information was available had fecal material in the bowel at surgery, and the distribution was fairly even among the five test groups. There were two instances of gross peritoneal contamination at surgery, one of which was followed by a wound infection with Escherichia coli (in group 58A 237).

Figure 1 illustrates the percentage of infections related to surgery in each of the groups. Table 1 indicates the number of different types of infection by site. Wound infections were the most common type. All cases of enterocolitis were due to S. aureus. In Table 2 the organisms responsible for infections related to surgery are shown.

Table 3 lists all patients who received chemotherapy other than the test drugs within a period of 7 days preoperatively or postoperatively. Any complications in such patients are noted so that the influence of nontest drugs may be evaluated.

Table 4 records the total deaths and indicates the number in which infection played a role in each group.

Side effects. The incidence of side effects such as epigastric burning, abdominal cramps, or frank diarrhea was minimal, and there were no distinct differences between the different drug groups.

Bacteriological data. Where the use of antibiotic or other preparations besides the test drugs might have influenced the fecal bacteriology, these results are eliminated from consideration.

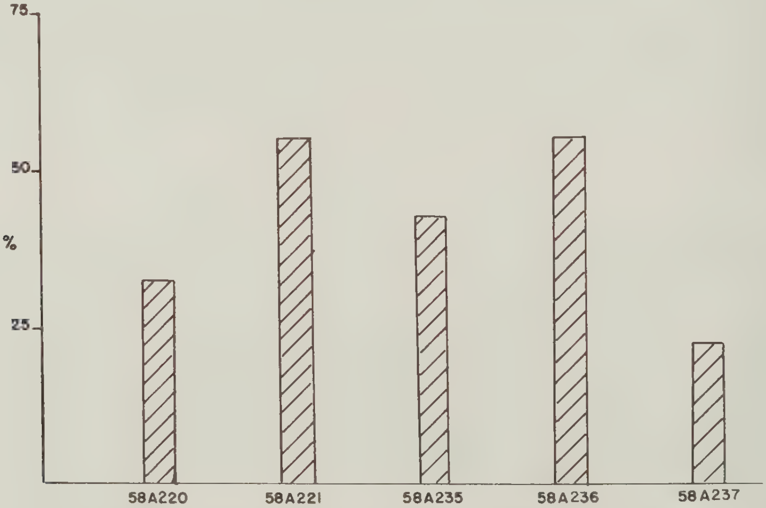

Fig. 1 Total patients with postoperative infection related to surgery.

Table 1. Postoperative Infections by Site

Drug	Number of patients	Related to surgery					Unrelated to surgery
		Wound	Peritonitis	Perineal infection	Enterocolitis	Miscellaneous infections	
58A220	18	4	0	2	1	0	2
58A221	20	7	0	2	1	1	2
58A235	18	2	2	3	2	1	2
58A236	19	7	0	3	0	1	2
58A237	17	2	2	0	0	1	4

Table 2. Postoperative Infections by Organisms*

Drug and number of patients	Wound infections					Other infections related to surgery				
	S. aureus†	E. coli‡	Other GNR§	Mixed‖	Misc.#	S. aureus†	E. coli‡	Other GNR§	Mixed‖	Misc.#
58A220 18	3	0	0	1	0	1**	0	0	0	2
58A221 20	3	3	1	1	1	2 (1**)	1	0	0	2
58A235 18	2	0	0	0	0	4 (2**)	1	0	1	2
58A236 19	6	2	1	0	1	0	1	1	1	0
58A237 17	0	1	0	1	0	0	0	0	2	0

*In cases of mixed infections involving both S. aureus and Gram-negative bacilli, each was listed separately.

†S. aureus (coagulase-positive)

‡Plus other Escherichia species

§GNR, Gram-negative rods

‖Escherichia and other Gram-negative rods

#Miscellaneous and unknown organisms

**Enterocolitis

Table 3. Relation of Chemotherapy Other Than Bowel "Prep" to Complications in the 7 Day Pre- or Postoperative Period

Patient	Additional chemotherapy	Complication	Organism
A. 58A220 Group			
1	Sulfasuxidine 1 day prior to test drug	None	–
2	Neomycin, orally, for 1 day prior to test prep	None	–
3	Tetracycline orally for 7 days just prior to test prep	Enterocolitis postoperatively	S. aureus
4	Tetracycline discontinued 6 days prior to test drug; tetracycline postoperatively for 2 days	Wound infection and intra-abdominal abscess	E. Coli Proteus vulgaris
5	Tetracycline postoperatively for 2 days	Pneumonia (Third postoperative day)	S. aureus
B. 58A221 Group			
1	Neomycin, orally, 1 day prior to test prep	None	–
2	Chloramphenicol started 6 days postoperative because of fever	None (Chloramphenicol used for treatment of intra-abdominal infection)	
3	Penicillin and streptomycin started day after surgery	Enterocolitis	S. aureus
4	Penicillin and streptomycin started immediately postoperatively	Stitch abscess	Pseudomonas aeruginosa
5	Tetracycline for 1 day, then sulfamethoxypyridazine started immediately following surgery	None	–
6	Tetracycline for 3 days starting 4 days postoperatively for presumptive infection (not established)	None	–

Table 3 cont.

Patient	Additional chemotherapy	Complication	Organism
7	Chloramphenicol immediately following surgery for 3 days	None	–
8	Chloramphenicol immediately following surgery for 2 days	None	–
C. 58A235 Group			
1	Tetracycline for 1 day postoperatively	None	–
2	Tetracycline immediately following surgery	Enterocolitis (and evisceration)	S. aureus
3	Chloramphenicol for 1 day postoperatively	Wound infection	S. aureus
4	Chloramphenicol for 4 days postoperatively	Wound infection	No culture
5	Chloramphenicol, erythromycin, and vancomycin postoperatively	None (These drugs were used to treat widespread staphylococcal infection)	
D. 58A236 Group			
1	Sulfisoxazole given concurrently with test prep (for presumed urinary infection)	Wound infection	S. aureus
2	Sulfamethoxypyridazine preoperatively and penicillin plus streptomycin postoperatively	None	–
3	Sulfamethoxypyridazine with last day of test prep, chloramphenicol postoperatively for 4 days	Perineal drainage	Not cultured
4	Chloramphenicol postoperatively for 7 days	None	–
5	Chloramphenicol postoperatively for 8 days (for pelvic abscess due to Bacteroides found at surgery)	Wound infection	S. aureus P. aeruginosa
6	Penicillin and streptomycin for 5 days and chloramphenicol for 3 days postoperatively	Perineal drainage	S. viridans P. aeruginosa
7	Penicillin and streptomycin postoperatively, continued for 3 weeks	Wound infection	S. aureus anaerobes
8	Penicillin and streptomycin for 11 days starting on 2 days postoperative, sulfisoxazole for 4 days postoperative	Wound infection	S. aureus
E. 58A237 Group			
1	Penicillin and streptomycin postoperatively for 10 days	None	–

Table 5 indicates, in those patients who gave stool specimens at appropriate times, the amount and speed of reduction of the normal aerobic flora and the entire aerobic flora with the various drug regimens. Similar information is given graphically in Figs. 2, 3, and 4. Fig. 2 shows the cumulative percent of patients with normal aerobic flora (Escherichia sp., enterococci, and other streptococci) reduced below 1,000 organisms/g of wet stool at the indicated time intervals. The pretreatment cultures showed normal aerobic flora present to the extent of 10^8 to 10^{10} organisms per/g of stool in virtually all cases. Thus, a count of less than 1,000 represents a 99.999 to 99.99999% reduction. Approximately 90% of patients in the groups receiving drugs had this degree of reduction of normal

Table 4. Deaths in Each Drug Group

Drug number	Patients, operated or not	Total	Infections contributing
58A 220	20	1	1*
58A 221	20	–	–
58A 235	20	2	1
58A 236	19	2	–
58A 237	19	5	2†

*Infected prior to surgery.

†One of these patients had an abdomino-perineal pull-through operation with probable impairment of blood supply at the anastomosis site; second patient had perforation of colon due to carcinoma and was infected prior to surgery.

Table 5. Reduction in Aerobic Fecal Flora with Administration of Various Test Preparations

Drug	Number of patients	Number of patients with normal flora, <1000‡			Number of patients with entire flora, <1000			Number of patients with no aerobic growth		
		24 hr or less	25-48 hr	49-72 hr	24 hr or less	25-48 hr	49-72 hr	24 hr or less	25-48 hr	49-72 hr
58A220	19	7	6(4*)	4(4*)	3	3(3*)	4(4*)	0	3(2*)	2(2*)
58A221	18	6	9(6*)	1	3	4(2*)	1	2	2(2*)	2(1†)
58A235	19	3	13(6*)	2	1	3(2*)	2	1	2(1*)	1
58A236	19	7	6(1*)	4(4*,2†)	5	3(1*)	1(1*,1†)	5	3(1*)	1(1*,1†)
58A237	19	0	0	0	0	0	0	0	0	0

*First specimen not obtained until this time interval; reduction of fecal flora might have been more rapid, therefore.

†>72 hours

‡*E. coli*, enterococci, other streptococci

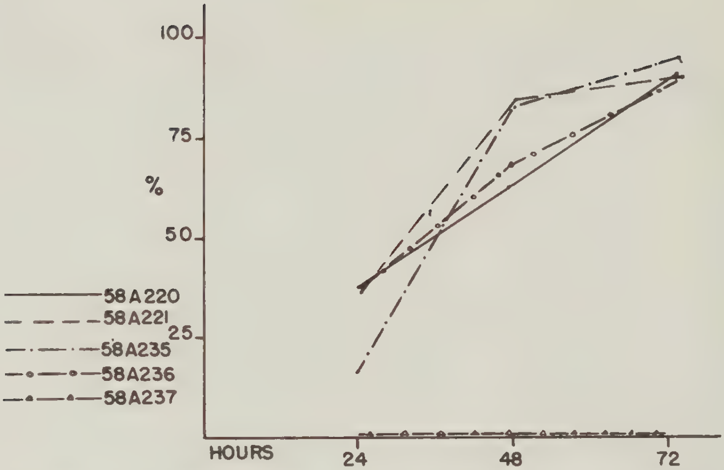

Fig. 2. Cumulative percentage of patients with normal aerobic fecal flora reduced to <1000.

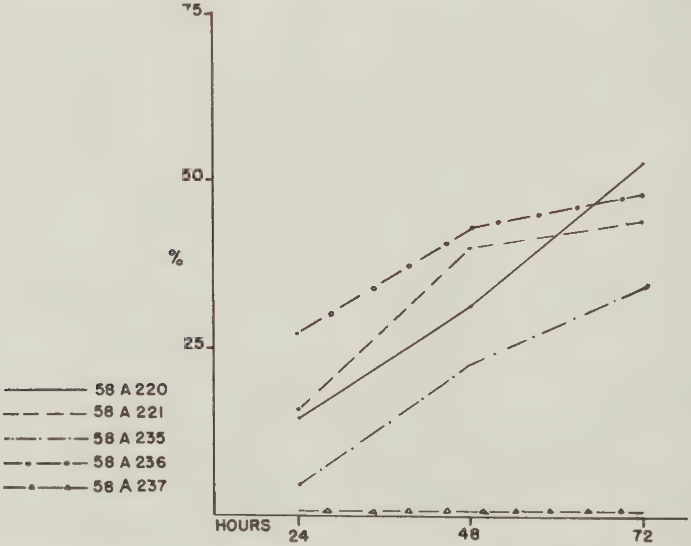

Fig. 3. Cumulative percentage of patients with entire aerobic fecal flora reduced to <1000.

aerobic flora by the third day of therapy. Figure 3 is similar, but records information on the entire aerobic flora (including such organisms as Klebsiella, Pseudomonas, S. aureus, etc. not normally found in large numbers in the stool). Only $^1/_3$-$^1/_2$ of the patients had their entire aerobic flora reduced to less than 1000/g after days of therapy, excluding the placebo group. Figure 4 gives the percentage of each drug group whose aerobic fecal flora was completely eliminated at the time of maximum effectiveness.

The "abnormal" aerobic fecal flora (organisms other than Escherichia, enterococci, and other streptococci) acquired as a result of administration of the various test preparations is recorded in Tables 6 and 7. Table 6 gives this information with respect to the time of maximum effect, if any, of the drug. Table 7 considers changes occurring within 10 days of discontinuing treatment with the test preparations before the normal flora returned. The smaller number

Table 6. "Abnormal" Aerobic Fecal Flora Acquired as a Result of Use of Various Test Preparations A. At Time of Maximum Effect of Drug

Drug	Number of patients	Coagulase-positive S. aureus		Klebsiella-Aerobacter		Pseudomonas		Yeast	
		10^5/g or less	>10^5/g	10^5/g or less	>10^5/g	10^5/g or less	>10^5/g	10^5/g or less	>10^5/g
58A 220	20	2	0	0	0	3	1	6	2
58A 221	18	0	0	0	1	6	0	6	0
58A 235	19	5	3	3	0	4	0	3	1
58A 236	19	2	0	0	2	5	1	3	1
58A 237	19	0	0	0	1	0	0	0	0

Table 7. "Abnormal" Aerobic Fecal Flora Acquired as a Result of Use of Various Test Preparations B. Within 10 Days After Discontinuing Therapy

Drug	Number of patients	Coagulase-positive S. aureus		Klebsiella-Aerobacter		Pseudomonas		Yeast	
		10^5/g or less	>10^5/g	10^5/g or less	>10^5/g	10^5/g or less	>10^5/g	10^5/g or less	>10^5/g
58A 220	14	1	7	1	2	3	1	1	1
58A 221	14	1	4	1	2	1	1	0	0
58A 235	11	0	8	2	3	4	0	0	1
58A 236	11	1	2	1	4	2	0	1	0
58A 237	7	0	0	0	1	1	0	0	0

*Results not included if other antibiotics given in this period.

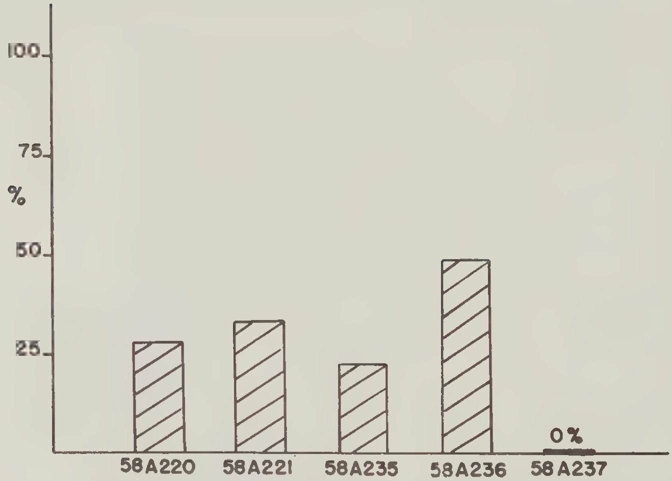

Fig. 4. Percentage of patients with aerobic fecal flora completely eliminated.

of patients in the latter table reflects the relative lack of postoperative stool specimens and the elimination of some results because of patients receiving other therapy which would modify the stool bacteriology. Most strains of S. aureus isolated either prior to or after discontinuing the test preparation were highly resistant to kanamycin and neomycin.

Figure 5 shows the incidence of coagulase-positive staphylococci in the stool by drug, regardless of whether these organisms appeared in the stool while the patient was receiving the drug, within 10 days of discontinuing the drug, or both.

Finally, Fig. 6 shows the correlation between S. aureus in the stool and the incidence of postoperative infections due to this organism.

There were insufficient postoperative stool specimens to determine exactly how long it took for the normal fecal flora to be re-established after therapy was stopped. In general, however, it usually took 7 to 10 days or longer for E. coli counts to reach 10^5/g of wet stool or higher. There were no particular differences noted between the four drug groups in this regard.

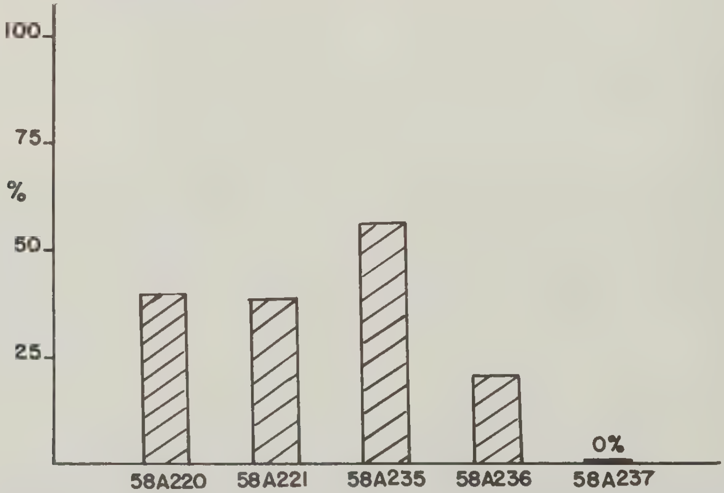

Fig. 5. Incidence of coagulase-positive S. aureus in stools (within 10 days of discontinuing drug).

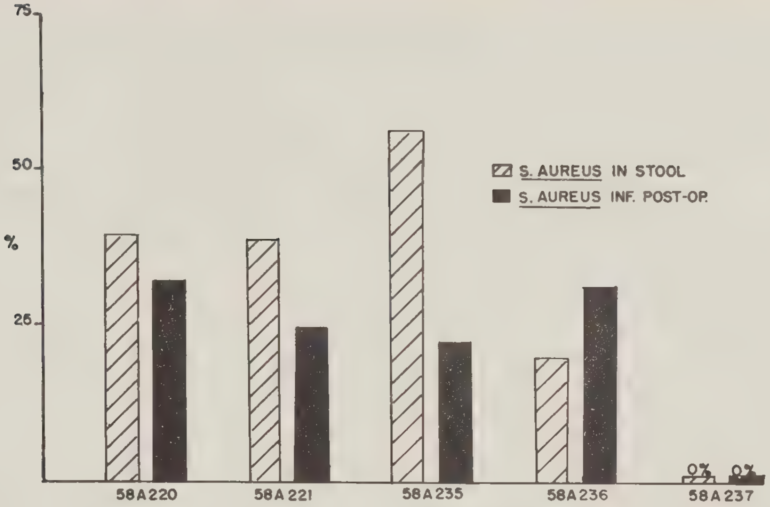

Fig. 6. Correlation of S. aureus in stool and S. aureus infections postoperatively.

DISCUSSION

Aside from the preponderance of younger patients in drug program 58A 236, the five groups of patients were quite comparable in all respects. One other factor to be considered in comparing results from one group to another is the matter of adjunctive chemotherapy either pre- or postoperatively (Table 3); the 58A 236 group had more postoperative infections which may have been related to such therapy than the other groups.

A comparison of postoperative infections in the four groups presumed to represent the drug programs indicates relatively minor differences, particularly considering the small number of subjects in each group. The 58A 220 group had the lowest incidence of infections related to surgery, but had the highest percentage of S. aureus infections. Postoperative infections related to surgery occurred with approximately the same frequency in the 58A 221 and the 58A 236 groups, with a somewhat smaller percentage in the 58A 235 group.

The correlation between the incidence of S. aureus in stools and the incidence of S. aureus infections postoperatively was fairly good except in the 58A 235 group. Staphylococcal enterocolitis occurred in all groups except the 58A 236 group and what is assumed to be the placebo group (58A 237).

Bacteriological comparisons do not suggest major differences among the four drug groups. The four drug programs appeared to have the same ultimate effectiveness as regards the reduction of the normal aerobic fecal microflora. Differences in rapidity of such reduction are difficult to interpret since on many occasions (Table 5) the first fecal specimen was not obtained until after more than 1 or 2 days of therapy had been completed. Nevertheless, with drug programs 58A 235 and 58A 236 there was definitely a number of patients whose fecal flora was not reduced maximally until the second day of therapy.

Marked reductions of the entire aerobic microflora (Table 5 and Fig. 3) were less consistently achieved indicating ingrowth of "abnormal" aerobic flora such as S. aureus, Klebsiella, etc; this is borne out further by the data in Table 6. Preparation 58A 235 is seen (Fig. 3) to have less effect on the entire aerobic flora than the other compounds; this is related to a higher rate of colonization with S. aureus (Fig. 5). Patients on preparation 58A 236 had the lowest incidence of S. aureus in their stools (pre- and postoperative periods considered together) and had the highest percentage of stools with no aerobic growth whatever in the preoperative period.

Not only is there ingrowth of "abnormal" flora resistant to the bowel "sterilizing" agents while they are being administered, but also there is a considerable amount of such colonization in the posttreatment period before the normal flora can grow back in (Table 7). This is especially striking in the case of S. aureus. These organisms need not be resistant to the agent used previously as shown by Dearing and Needham [7].

As indicated earlier, the differences in clinical and bacteriological results between the four programs thought to be the different dosage regimens of oral kanamycin and neomycin are relatively minor and cannot be given too much weight since only small numbers of subjects are involved. What is really striking is the difference between the results in what appears to be the placebo group and the drug groups. There was not a single recovery of S. aureus from stool cultures or from postoperative infections directly related to the surgery performed in the case of the placebo group (compared to a minimum of four cases of S. aureus infections in each of the other groups). S. aureus infections at distant sites (one furuncle and one pneumonia with septicemia) did occur in two patients in the 58A 237 group.

Furthermore, the placebo group's over-all infection rate (relating to surgery) was the lowest of all groups although the 58A 220 group was close. Disregarding S. aureus infections entirely, the 58A 237 group had an infection rate comparable to two of the other groups (58A 220 and 58A 235) and better than that of the other two. Certainly, therefore, the patients receiving the placebo did not have any undue risk of infection with Gram-negative bacilli or streptococci which might normally be present in the stool.

We have already commented on the lack of S. aureus in the stools of patients on 58A 237. Tables 6 and 7 indicate that this group was also less likely to acquire such other "abnormal" organisms as Klebsiella-Aerobacter, Pseudomonas, and yeast. This is an additional potential advantage of not using preoperative bowel "sterilization" since E. coli infections would ordinarily be much easier to manage than infections due to such resistant organisms as Klebiella-Aerobacter and Pseudomonas.

Also, it is well to remember that neomycin and kanamycin are quite inactive against most anaerobes so that infections with Bacteroides, Clostridium, and anaerobic cocci are potential dangers when these agents are used.

The situation at Wadsworth Veterans Hospital is unusual since the staphylococci predominating in our infections, and in our environment as well, are of phage type 54; most of the strains of this phage type are highly resistant to kanamycin and neomycin (as well as to paromomycin and chloramphenicol). The resistance is so great that these organisms may implant and set up enterocolitis in spite of the high fecal drug levels achieved with oral administration kanamycin or neomycin [8]. With this background, it is not surprising that there should be such a strking incidence of S. aureus in the stools and in postoperative infections in patients receiving these agents. This problem of neomycin and kanamycin-resistant staphylococci may become even more widespread and important. We have recently encountered staphylococci of two other phage types which are resistant to these agents [9] and such resistant organisms are being found in a number of other localities [10-12]. It has already been pointed out that staphylococci sensitive to these drugs may colonize the intestinal tract after therapy is stopped and set up infection [7] so that S. aureus remains a potential threat in patients receiving such agents even when there is no problem of antibiotic resistance. Since our data suggest that a period of 7 to 10 days or longer following discontinuation of therapy is frequently necessary before E. coli again reaches counts of 10^5/g of wet stool, there would seem to be ample opportunity for implantation of antibiotic-sensitive staphylococci.

In the light of the favorable results obtained in the placebo group in this study, should we not reconsider the advisability of preoperative bowel "sterilization" as suggested by Tyson and Spaulding [3] and others? Let us examine again the evidence which seemed to indicate the desirability of such preoperative preparation. The two major points which seem to stand out are the clinical impression that patients prepared with chemotherapeutic agents are better off because of this, and experimental evidence indicating greater stability of colonic anastomoses when protected with antibiotics. In the absence of definitive data from controlled series documenting the former point, it would seem reasonable to question the validity of these clinical impressions in the light of the results of our small, controlled, double-blind series. Adequate controls are necessary to avoid bias or misinterpretation relating to patient selection, supportive therapy, use of other drugs parenterally, and to mechanical cleansing of the bowel alone. Recently Borgstrom and Sandberg [13] have questioned the evidence regarding greater stability of colonic anastomoses on the basis of studies in rabbits whose aerobic intestinal microflora was reduced by the use of antibiotics.

Certainly our series is far too small to justify any definitive conclusions. Nevertheless, it is now reasonable to seriously question the desirability of routinely using chemotherapeutic agents as part of the procedure for preoperative bowel preparation.

SUMMARY

A double-blind study employing two different dosages of oral kanamycin and of oral neomycin and a placebo in five groups of 20 patients each has been performed. Results of bacteriological studies and analysis of postoperative infections are given. The most striking result is the favorable outcome in the placebo group, both with regard to incidence and type of postoperative infection and the stool bacteriology. The desirability of routine preoperative bowel "sterilization" must be seriously questioned.

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A CONTROLLED BLIND STUDY OF THE EFFECTS OF ANTIBIOTICS ON THE GASTROINTESTINAL TRACT*

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INTRODUCTION

The effects of antibiotics on the fecal flora have been described by many authors [1-7] and the gastrointestinal problems related to antibiotic therapy are well known. To the best of our knowledge there have been no studies to correlate the effects of antibiotics on the gastrointestinal mucosa with respect to observations for cytological changes and sigmoidoscopic evaluation. Our study was performed to correlate the effects, in a controlled blind fashion, of antibiotics on the gastrointestinal tract of healthy volunteers in order to ascertain whether these effects could be attributed to the antibiotics or to alterations of the intestinal microorganisms.

METHODS

The results of observation of fifty healthy volunteers are reported in this paper. The agents employed were placebo, erythromycin base, propionyl erythromycin ester lauryl sulphate, tetracycline, and tetracycline plus stress-formula vitamins.† Each subject received two 250-mg capsules of one of the above agents q.i.d. for a period of seven days. All the medications were prepared in identical capsules and labeled only with code numbers. The nature of therapy was unknown both to subject and to observers. Each subject had a history and physical examination prior to therapy. Sigmoidoscopic examinations on the unprepared bowel were performed prior to, one or more times during, and immediately after therapy. In addition, another sigmoidoscopic examination was performed several days following cessation of all treatment. At the time of each sigmoidoscopic examination a direct smear was made from the bowel wall for cytological evaluation. Eight stool specimens were also secured at this time. In addition, the subjects collected stools at regular intervals during the three phases of the study. Each fecal specimen was evaluated by smear, Wright's count, culture and identification of the flora, parasitological examinations, and mycological examinations. In addition, in vitro sensitivity studies with the antibiotics were performed on representative cultures from the following large microbiological groups: coli-aerogenes, micrococci, Proteus, and Pseudomonas.

RESULTS

Pretreatment control studies failed to reveal any significant abnormalities in any of the areas noted above. Table 1 summarizes the clinical changes induced

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*This study was made possible by a grant from the Eli Lilly Laboratory for Clinical Research.

†These preparations, including the placebo, were obtained through the courtesy of Eli Lilly Laboratory for Clinical Research.

Table 1. Symptoms Noted During the Administration of the Placebo and Antibiotics

Symptom	Placebo, 10 subjects	Erythromycin, 10 subjects	Erythromycin-lauryl sulphate, 6 subjects	Tetracycline, 12 subjects	Tetracycline plus stress-formula vitamins, 12 subjects
Upper gastrointestinal complaints	0	2	3	1	2
Abdominal pain	2	2	1	1	3
Soft stools	2	3	1	1	1
Diarrhea	0	1	0	1	2
Constipation	0	1	2	0	0
Anal pruritis	0	2	1	0	1
No complaints	8	5	2	7	5

Table 2. Sigmoidoscopic Changes in the Lower Colonic Segment Observed Following the Administration of Placebo and Antibiotics

Change	Placebo, 10 subjects	Erythromycin, 10 subjects	Erythromycin-lauryl sulphate 6 subjects	Tetracycline, 12 subjects	Tetracycline plus stress-formula vitamins, 12 subjects
Mucus	1	0	1	4	5
Bowel-wall congestion	1	2	2	4	6
Anorectal-line inflammation	1	3	2	5	2
Perianal inflammation	0	0	1	4	1
Unchanged from control	9	6	4	4	6

Table 3. Cytological Changes* of the Bowel Wall Exudates Observed During the Administration of Placebo and Antibiotics

Change	Placebo, 10 subjects				Erythromycin, 10 subjects				Erythromycin-lauryl sulphate, 6 subjects				Tetracycline, 12 subjects				Tetracycline plus stress-formula vitamins, 12 subjects			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Mucus	–	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mucus and epithelium cells	3	2	–	–	2	1	1	–	–	–	–	–	–	2	1	4	–	5	2	1
Mucus, epithelium, and inflammatory cells	2	2	–	–	–	–	1	3	2	–	2	2	–	1	–	2	1	–	–	3
No cells observed	–	–	–	–	1	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–

*Changes are graded from 1 to 4 in order of ascending severity.

Table 4. A Comparison of the Changes in the Number of Organisms from the Control Cultures to the Stool Cultures After Therapy

Organism	Placebo, 10 subjects		Erythromycin, 10 subjects		Erythromycin-lauryl sulphate, 6 subjects		Tetracycline, 12 subjects		Tetracycline plus stress-formula vitamins, 12 subjects	
	Increase	Decrease	Increase	Decrease	Increase	Decrease	Increase	Decrease	Increase	Decrease
Coliforms	0	0	0	4	3	0	0	7	1	7
Proteus	0	3	4	0	2	0	8	0	8	0
Streptococci	1	1	1	4	3	1	6	0	4	1
Staphylococci	1	3	7	0	4	2	5	1	6	0
Yeasts	1	0	2	0	2	1	8	0	5	0

by the agents studied. The placebo group is essentially unchanged from pretreatment observations. There occurred in the erythromycin treated groups an increase in the number of essentially well-formed stools associated with mild abdominal pain. Diarrheal stools were not prominent in this group. However, they become more prominent in the tetracycline treated group. It must be noted that two subjects taking erythromycin noted cramping severe enough to discontinue the agent. Two subjects taking tetracycline and one subject taking tetracycline with stress-formula vitamins were forced to discontinue therapy due to the severity of the diarrhea. Observations on these subjects were continued and are reported in our results.

Table 2 is a summary of the sigmoidoscopic observations following the administration of the placebo and the antibiotics. The placebo group revealed little or no change from the pretreatment observations. The groups treated with erythromycin and propionyl erthromycin ester lauryl sulphate were relatively unchanged except for some evidence of mild anorectal-line inflammation and minimal mucosal congestion. Tetracycline and tetracycline stress-formula groups were marked by an increase in mucus, intense congestion of the bowel wall, and moderately severe anorectal-line inflammation.

Table 3 is a comparative study of the effects of antibiotics on the intestinal mucosa as reflected in the cytological changes in material removed directly from the rectal wall. The mildest change consisted of the production of large amounts of mucus. Increasingly severe reactions coincided with the appearance of epithelial cells in addition to mucus and fibrin. The more severe reactions were marked by the appearance of various inflammatory cells in addition to the elements noted above. The cell pictures were graded from one to four plus in order of ascending severity. The placebo produced only the mildest cytological changes. Erythromycin and propionyl erythromycin ester lauryl sulphate produced moderate degrees of cytologic changes with the appearance of inflammatory cells in a number of cases. The most severe reactions, however, appeared in the tetracycline and tetracycline plus stress-formula vitamin-treated groups.

Table 4 records the bacteriological changes produced in the fecal flora by each of the agents. The placebo group showed little change from the control cultures. Erythromycin produced an increase in the staphylococci and an increase in Proteus. Tetracycline-treated groups were characterized by an increase in Proteus, staphylococci, and yeasts. The coliforms were significantly decreased.

Parasitological observations were made on all stool specimens and Endamoeba histolytica cysts were found in one of the tetracycline subjects, disappearing during treatment and reappearing in the posttreatment specimens.

In vitro sensitivity studies revealed the greatest increase in resistance to have occurred to tetracycline and tetracycline plus stress-formula vitamins in staphylococci and coli-aerogenes groups.

DISCUSSION

It appears from these data that no one specific microorganism is responsible for the changes observed during the administration of the various antibiotics. In all the subjects complaining of increased intestinal motility in which gross mucosal changes and cytological reactions were observed, stool cultures revealed an increase in staphylococci, Proteus, or yeasts. Tetracycline and tetracycline plus stress-formula vitamins produced the most striking effects in all areas of observation.

SUMMARY

Erythromycin base, propionyl erthromycin ester lauryl sulphate, tetracycline, tetracycline plus stress-formula vitamins, and a placebo were evaluated in a group of fifty healthy volunteers by blind technique. Tetracycline and tetracycline with stress-formula vitamins revealed an increase in both subjective and objective effects on the intestinal tract more intense than in either the placebo or erythromycin treated subjects.

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DISCUSSION

DR. COTLAR: I would like to congratulate Dr. Epstein on this study, particularly on being able to get 56 medical students to submit to this in view of the sigmoidoscopic examinations. Dr. Isidore Cohn in our department has been evaluating preoperative bowel preparations for the past few years and particularly within the last two years has been evaluating the use of a neomycin-achromycin combination. He has found that nausea, vomiting, and gastrointestinal disturbances are not uncommon and frequently these reactions begin very early. When the patients are switched to kanamycin the gastrointestinal disturbances disappear.

COLISTIN IN THE TREATMENT OF HOSPITALIZED PATIENTS WITH PSEUDOMONAS INFECTIONS

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INTRODUCTION

The past few years have seen the emergence of Pseudomonas infections as an increasingly troublesome cause of morbidity and mortality among hospitalized patients [1, 2]. Polymyxin alone has consistently shown good in vitro activity against these organisms, but its use in patients has frequently been attended with troublesome neuro- and nephrotoxicity, and questionable therapeutic results. Recently, colistin, a new antibiotic which is similar to polymyxin in antibacterial spectrum and chemical structure, has become available. Originally developed in Japan, colistin was more recently introduced into this country where laboratory and clinical studies over the past two years have shown it to be less toxic than polymyxin, although having comparable antibacterial activity [3, 4]. The present report describes the results of colistin therapy in 28 patients with Pseudomonas infections treated at the University of Washington Affiliated Hospitals between August, 1959, and October, 1960. Data on the in vitro susceptibility of hospital strains of Pseudomonas are also presented.

METHODS AND MATERIALS

Only patients with clinically significant Pseudomonas infections were selected for treatment. The Pseudomonas strains were identified by typical colonial appearance and pigment production. All were tested by the single disk method for susceptibility to tetracycline, chloromycetin, streptomycin, polymyxin, sulfa drugs, neomycin, kanamycin, and nitrofurantoin. The methylated form of colistin, colistimethate sodium,* was supplied as a sterile white powder in vials containing 16, 30, or 150 mg, with 1, 2, or 8 mg of dibucaine hydrochloride, respectively. In all but one instance the drug was diluted in sterile water for injection and administered intramuscularly. In one patient, an infant with aplastic anemia and a marked bleeding tendency, 16 mg of the antibiotic were diluted in 200 ml of a 5% dextrose aqueous solution and given intravenously over an 8 hr period. The dosage employed early in the study was generally about 2 mg/kg/day. Later, when data on serum antibiotic levels became available, doses of 4–10 mg/kg/day were employed in some patients. The daily dose was usually administered in four intramuscular injections given 6 hr apart but some patients with poor renal function received injections only every 12 hr. In a few patients serum levels of colistin were determined using a twofold tube dilution method and colistin sulfate standard. The assay organism, Bordetella bronchiseptica ATCC 4617, was usually inhibited by 0.05 μg/ml of colistin base as colistin sulfate. In addition to blood level determinations, the ability of the patient's serum to inhibit growth of the infecting Pseudomonas was determined in some instances using a twofold serial dilution of serum and an inoculum of about 250,000 organisms/ml.

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*The trade name of Warner-Chilcott Company for colistimethate sodium is Coly-mycin M. Vial contents are equivalent in activity to the labeled amounts of colistin base.

Pseudomonas isolates from 30 hospitalized patients were tested for susceptibility to colistin by a tube-dilution method using colistin sulfate standard.* The comparative activities of colistin sulfate and colistimethate sodium were determined by simultaneous assays with 12 Pseudomonas isolates. For these tests stock solutions were prepared in distilled water for each form of colistin, with activity equal to 1,000 μg of colistin base per ml. Further dilutions were then made in tryptose phosphate broth to give final concentrations of 32, 16, 8, 4, 2, 1, 0.5, and 0.25 μg/ml. The inoculum consisted of 1–5 million organisms per milliliter from an 18-hr broth culture. The lowest concentration of antibiotic producing complete inhibition was determined by inspection after overnight incubation. Comparative bactericidal activity was estimated by subculturing a loopful of broth onto agar from each of the tubes showing no growth. The tests were performed on three different days with a separate preparation of stock solutions for each determination and the arithmetic mean for each isolate was then computed.

RESULTS

Susceptibility tests. Results of the disk susceptibility tests of the infecting Pseudomonas were available for 26 of the 28 cases. Two of the 26 were resistant to all the antibiotics used for testing. Fourteen were susceptible only to polymyxin and eight were susceptible only to neomycin and polymyxin. Some of these isolates were included in a separate study of 30 strains tested by the tube-dilution method. All 30 isolates were inhibited by from 1 to 5 μg/ml of colistin base as colistin sulfate.

The results of the simultaneous susceptibility tests using the two forms of colistin, colistin sulfate, and colistimethate sodium are shown in Table 1. The minimal inhibitory concentrations are expressed in terms of "base equivalents," each figure representing the concentration of colistin base giving activity against

Table 1. The Comparative Activity of Colistin Sulfate and Colistimethate Sodium Against Pseudomonas Isolated from Hospitalized Patients

Isolate	MIC*		MCB*	
	Sulfate	Colistimethate	Sulfate	Colistimethate
1	0.8	2.0	2.0	4.7
2	1.0	2.7	2.0	16.0
3	1.0	4.7	3.0	8.0
4	0.5	2.7	1.7	6.7
5	1.3	4.7	4.7	13.3
6	1.1	2.7	2.7	10.7
7	0.7	2.3	1.3	4.0
8	1.3	5.3	16.0	26.7
9	3.3	10.7	9.3	32.0
10	1.3	2.7	3.3	6.7
11	1.7	3.3	8.0	8.0
12	0.5	2.7	4.7	10.7

*The minimal inhibitory concentrations and the minimal bactericidal concentrations are expressed as μg of colistin base/ml.

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*Standard powders were kindly provided by the Warner-Lambert Research Institute, Morris Plains, New Jersey. The colistin sulfate was labeled WL-31859, with potency of 666 μg colistin base/mg, and the colistimethate sodium was labeled MCB 2059, with potency of 341 μg colistin base/mg.

B. bronchiseptica ATCC 4617 equivalent to the concentration of the colistin analogue being used in the test. The data show that under the conditions of this test one base equivalent of colistimethate sodium is ½–⅕ as active against Pseudomonas as one base equivalent of colistin sulfate. The average minimal inhibitory concentration of the colistimethate was 3.9 μg/ml and ranged from 2.0 to 10.7 μg/ml.

Serum concentrations. The results of determinations of serum levels of colistin are presented in Table 2 for six patients. For case 26, the level represents the average of three determinations, and for cases 19, 17, and 15, two determinations, while for cases 8 and 1 values represent single determinations. None of the colistin levels exceeded 6.7 μg/ml. Cases 17, 1, and 15 had tests of serum activity against small inocula of their infecting Pseudomonas. Cases 17 and 1 both had markedly impaired renal function and received only 1.7 mg/kg/day of colistin. Serum from case 1, 12 hr after receiving an injection of 0.85 mg/kg of colistin, inhibited the infecting Pseudomonas at a serum dilution of 1 : 16. A higher single dose, 1.9 mg/kg, in case 15, a patient with a BUN of 14, also produced a good inhibitory level (1 : 8) in serum 6 hr later.

Table 2. Levels of Serum Activity After Intramuscular Injections of Colistin

Case number	Renal function before therapy	Dose, mg	Hours since last injection	Serum level, μg/ml*	Maximum serum dilution inhibiting the patient's Pseudomonas
26	BUN† 15	16.6	1	3.9	
8	BUN 14	16.6	1	6.7	
			3	3.3	
			5	3.3	
19	BUN 29	16.6	1	5.4	
			5	3.5	
17	Creatinine 5.6	33.3	1	3.3	1:8
			3	6.7	1:8
1	Creatinine 6.8	33.3	6		1:16
			12		1:16
15	BUN 14	150	6		1:8

* μg/ml of colistin base as colistin sulfate.
†BUN, Blood Urea Nitrogen, mg/100 ml

Clinical data and response to therapy. Clinical summaries of the 28 cases with Pseudomonas infections are presented in Tables 3, 4, and 5. The patients ranged in age from 20 days to 74 years. Sixteen were males and twelve were females. Many had serious underlying diseases which might be expected to increase their susceptibility to infection. Sixteen had chronic urinary tract infections, six had septicemia including two with proven endocarditis, five had pulmonary infections, and one had acute cystitis.

The results of treatment are presented in Table 6. Of the 27 cases with sufficient diagnostic and follow-up data to make a clear judgment, twelve showed bacteriological cure. An additional eight cases showed definite clinical and bacteriological improvement, and there were seven failures. In one case the result of therapy was indeterminate.

Colistin was highly effective in the 17 patients with urinary tract infections. Ten were cured, and six were unquestionably improved. In the remaining case,

Table 3. Clinical Summaries of Twelve Cases of Pseudomonas Infections Cured by Colistin Therapy

Diagnosis	Age and sex	Weight, kg	Renal status	Therapy	Result
1. Polycystic kidneys, uremia, hypertension, pyelonephritis. Pseudomonas sensitive only to polymyxin.	38 F	39	Creatinine 6.8	33 mg q. 12 h., 7 days	Pseudomonas eliminated and pyuria cleared completely. No return of pyuria or Pseudomonas 24 days after stopping therapy.
2. Reduplication of renal collecting system, with recurrent urinary tract infections. Pseudomonas sensitive to polymyxin only.	2½ F	14.5	BUN* 15	15 mg q. 6 h., 13 days	Urine cleared of pyuria and Pseudomonas permitting successful surgical correction later on.
3. Hydronephrosis secondary to reduplicated renal collecting system. Suprapubic cystostomy. Pseudomonas sensitive to polymyxin and neomycin.	F	5.8	BUN 21	15 mg q. 6 h., 5 days	Urine sterilized. Pyuria cleared. No return of infection one week after colistin was stopped.
4. Brainstem thrombosis due to hypertensive vascular disease. Chronic pyelonephritis. Pseudomonas sensitive to polymyxin and neomycin.	42 M	81	BUN 16 PSP 43%	150 mg q. 6 h., 3 days, 150 mg q. 6 h., 2 days	Pyuria cleared, Pseudomonas eliminated from the urine. No pyuria and culture negative 2 weeks after colistin was stopped.
5. Probable chronic pyelonephritis since 1955. Nephrolithotomy, 1959. Nephrectomy, 1959. Bladder surgery, 1960. Urine showed pyuria and pure culture of Pseudomonas sensitive only to polymyxin.	68 M	66	BUN 27	75 mg q. 6 h., 5 days	No pyuria and culture of urine sterile at the end of therapy and 8 days later.

Table 3 cont.

Diagnosis	Age and sex	Weight, kg	Renal status	Therapy	Result
6. Bladder neck stricture, with chronic pyelonephritis requiring more than ten hospitalizations since 1949. Pseudomonas sensitive only to polymyxin.	74 M	?	BUN 31	30 mg q. 6 h., 9 days	Complete clearing of pyuria and Pseudomonas despite superinfection by Proteus After subsequent cystostomy, 2 weeks later, the patient continued to do well; four months after colistin was stopped the urinalysis was normal and BUN was 22.
7. Chronic pyelonephritis in sole remaining kidney scarred by old tuberculosis. Pseudomonas sensitive only to polymyxin.	44 F	59	Creatinine 1.0	50 mg q. 6 h., 5 days	Urinary infection cleared. At recurrence 3 months later only enterococci were isolated.
8. Chronic pseudomonas urinary tract infection following bladder catheterization during acute bulbospinal poliomyelitis one month previously. Pseudomonas sensitive only to polymyxin.	24 M	58	BUN 14	16 mg q. 6 h., 3 days	Gross pyuria and symptoms of dysuria and frequency cleared, and Pseudomonas was eliminated from the urine. Urinalyses negative with 1 year followup.
9. Bladder neck obstruction megacystis, with acute urinary tract infection following surgery on the bladder neck. Pseudomonas susceptible to polymyxin only.	39 M	73	BUN 20	75 mg q. 6 h., 5 days	Temperature of 103 F fell to normal. Gross pyuria cleared and the urine culture became negative.

Table 3 cont.

Diagnosis	Age and sex	Weight, kg	Renal status	Therapy	Result
10. Laennec's cirrhosis, chronic obstructive emphysema, bleeding gastric ulcer, acute urinary tract infection with $2 \cdot 10^6$ pseudomonas/ml. Pseudomonas susceptible to polymyxin only.	73 M	?	BUN 12	75 mg q. 6 h., 6 days	Afebrile after 3 days of therapy. Pseudomonas cleared from urine. No return of infection one week after therapy was discontinued.
11. Renal shutdown and other complications of gun shot wound of abdomen. Pseudomonas in wounds, tracheal secretions, urine and intermittantly in blood, sensitive to neomycin and polymyxin only.	33 M	66	BUN 66	16 mg q. 6 h., 19 days	Pseudomonas disappeared from wounds, trachea and urine. BUN fell spontaneously to 34 while the patient was being treated with colistin.
11a. Same patient 8 months later developed mixed pseudomonas-enterococcus septicemia and meningitis diagnosed by many positive cultures over a 5-day period. Sensitive to polymyxin and chloromycetin only.		43	BUN 22	75 mg q. 6 h., 14 days	Complete clearing of blood and cerebrospinal fluid of Pseudomonas. Patient also received penicillin and streptomycin for the enterococcus, and 2.5 mg polymyxin B intraventricularly for 10 days.
12. Prematurity. Tracheo-esophageal fistula. Right upper lobe pulmonary abscess secondary to aspiration. Pseudomonas susceptible only to polymyxin.	2 months F	2.2	BUN 16	5 mg q. 6 h., 21 days (Colistin was also applied topically into the abscess cavity.)	Abscess was finally sterilized with topical therapy and went on to heal favorably.

Table 4. Clinical Summaries of Eight Cases Clinically and Bacteriologically Improved by Colistin

Diagnosis	Age and sex	Weight, kg	Renal status	Therapy	Result
13. Spinal cord transection, T-8. Pseudomonas septicemia following grafting of a sacral decubitus ulcer, with fever spikes to 105 F and hypotension. Urinary tract infection. Pseudomonas sensitive only to polymyxin.	64 M	50	BUN 32	75 mg q. 6 h., 12 days	Blood cultures became sterile. Blood pressure rose to normal levels. Occasional fever spikes continued in association with a proteus-enterococcus urinary infection.
14. Chronic pyelonephritis with gross pyuria refractory to all treatment. Pseudomonas resistant to all antibiotics tested.	40 M	80	Creatinine 1.8	100 mg q. 8h., 7 days	Pyuria cleared. Methionine and mandelamine have maintained him with low colony counts and only scant pyuria for five months following colistin treatment.
15. Cystinuria with renal calculi. Chronic pyelonephritis. Pseudomonas sensitive only to polymyxin.	29 F	84	BUN 14	150 mg q. 12 h., 9 doses, 75 mg q. 6 h., 10 doses	Pyuria cleared despite superinfection with enterococci. Pseudomonas and pyuria returned after therapy was discontinued.
16. Paraplegia secondary to traumatic cord transection. Progressive pyelonephritis by intravenous transfusions since 1953. Pseudomonas sensitive to neomycin, streptomycin, chloromycetin, furadantin.	23 M	59	BUN 26	8 mg q. 6 h., 5 days, 16 mg q. 6 h., 1 day 30 mg q. 6 h., 7 days	Became afebrile and gross pyuria cleared. Cultures became overgrown with Proteus. Pyuria returned when colistin was stopped although only Proteus could be isolated from the urine. When acutely ill, had paraesthesias with 16 mg dose. Later, no side effects with 16 or 30 mg doses.

Table 4 cont.

Diagnosis	Age and sex	Weight, kg	Renal status	Therapy	Result
17. Chronic pyelonephritis in a solitary left kidney. Pseudomonas resistant to all antibiotics tested.	44 F	59	Creatinine 5.6	33 mg q. 6 h., 1 day, 33 mg q. 12 h., 7 days	Reduction in chronic infection, but Pseudomonas recurred in urine cultures when colistin was stopped.
18. Bilateral renal calculi since 1949 with chronic infection. Pseudomonas sensitive only to polymyxin.	39 F	61	Creatinine 1.7	75 mg q. 6 h., 6 days	Pyuria and colony counts fell to near zero, but full infection recurred when colistin was discontinued.
19. Debilitated patient with infected amputation stump, pseudomonas urinary tract infection, urethral abscess and wound infection. Pseudomonas sensitive to polymyxin and neomycin only.	50 M	23	BUN 29	16 mg q. 6 h., 25 days	Pyuria markedly reduced and urethral abscess healed after it was incised and drained. Pseudomonas persisted in the wound.
20. Mucoviscidosis, pneumonia. Pseudomonas sensitive to neomycin and polymyxin only	2½ M	10	BUN 20	15 mg q. 6 h., 5 days	Pure Pseudomonas was cleared from the nasopharyngeal culture with some clinical improvement. Coagulase-positive staphylococci then appeared and the pneumonia progressed. Responded to chloromycetin.

Table 5. Clinical Summaries of Seven Cases in Which Colistin Therapy Failed.

Diagnosis	Age and sex	Weight, kg	Renal status	Therapy	Result
21. Postoperative resection of an aortic aneutrism. Urine and blood cultures first positive for Pseudomonas sensitive only to polymyxin 9 days before treatment was started	74 M	69.5	BUN 25	75 mg q.6 h., 9 days	Fever and positive blood cultures persisted throughout therapy, although pyuria cleared and urine cultures were negative after the 2 days. Acutely inflamed gallbladder removed the day before death showed Pseudomonas on culture. Autopsy: bacterial endocarditis, blood culture positive for Pseudomonas.
22. Aplastic anemia, marked bleeding tendency, Pseudomonas septicemia. Pseudomonas sensitive only to polymyxin and neomycin.	2 F	?	BUN 11	16 mg in 200 cc q. 8 h, i.v. 3 doses	High spiking fevers and positive blood cultures persisted. Patient expired. Autopsy blood cultures showed Pseudomonas.
23. Mongolism, acute leukemia, Pseudomonas septicemia.	27 months M	10.8	?	20 mg	Patient expired 4 hr after the dose. Blood culture (at autopsy) still positive for Pseudomonas.

Table 5 cont.

Diagnosis	Age and Sex	Weight	Renal Status	Therapy	Result
24. Bronchopneumonia unresponsive to chloromycetin, erythromycin and mycostatin. Pseudomonas sensitive only to polymyxin and neomycin.	$2\frac{1}{2}$ months F	3.5	BUN 12	1.5 mg q. 6 h., 5 days	Heavy growth of Pseudomonas persisted in sputum, nose, and throat. X-ray worsened. Patient expired on fifth day of therapy.
25. Prematurity. Right middle lobe pneumonia. Pseudomonas sensitive to polymyxin, neomycin, streptomycin, and kanamycin.	20 days	1.5	BUN 16	0.75 mg q. 6 h., 32 days	Pure growth of Pseudomonas in nasopharyngeal culture gave way to mixed flora, but colonies of Pseudomonas persisted there and in gastric secretions. Patient also failed to respond to polymyxin or kanamycin and expired.
26. Chronic suppurative lung disease due to Pseudomonas. Long term prednisolone therapy for asthma.	39 F	45	BUN 15	16 mg q. 8 h., 9 days	Fever declined after 5 days of therapy, but Pseudomonas persisted in the sputum.
27. Congenital bladder neck obstruction, hydronephrosis, and chronic urinary tract infection. Pseudomonas sensitive only to polymyxin.	16 months M	9	BUN 11	15 mg q. 8 h., 6 days	Pyuria persisted throughout therapy. Pseudomonas in the urine was replaced by Proteus and staphylococci. Pseudomonas recurred in the urine cultures when colistin was stopped.

case 27, the Pseudomonas was suppressed by colistin but superinfection with Proteus and coagulase-positive staphylococci prevented a favorable clinical result.

Colistin was of questionable benefit in four of the five patients with pulmonary infections. Case 12, a premature infant, had a lung abscess which responded to topical instillations of the antibiotic. Of the remaining four cases, one was a child with mucoviscidosis and pneumonia, and a pure growth of Pseudomonas in the sputum (case 20). With colistin therapy 6 mg/kg/day there was some transitory clinical improvement and clearing of Pseudomonas from the sputum. However, superinfection with staphylococci occurred and the pneumonia progressed. The other three patients all received 1.7–2.4 mg/kg/day of colistin, and none showed clinical improvement or elimination of the infecting Pseudomonas.

Table 6. Results of Treatment in Twenty-Eight Patients with Pseudomonas Infections

Infection	Bacteriological cure	Clinical and bacteriological improvement	Treatment failure	Indeterminate
Urinary tract	10	6	1	
Pulmonary	1	1	3	
Septicemia	1	1	2	
Endocarditis	0	0	1	1

Four of the six cases of septicemia were treatment failures, but one of these received only a single injection of colistin before death. In another, an infant with aplastic anemia, colistin was given intravenously since a marked bleeding tendency prohibited intramuscular injections. Because of the rapid renal excretion of colistin in children, adequate blood levels may not have been attained. Of the two patients with proven endocarditis, case 21 failed to respond to treatment and expired. A well established urinary infection cleared rapidly in this man although blood cultures remained positive throughout the 9 day course of treatment with 4.3 mg/kg/day of colistin. The other patient with endocarditis (case 28) expired 6 hr after his first injection of colistin. A post-mortem blood culture was negative for Pseudomonas, and the result of therapy is considered indeterminate.

Side effects. There were 14 patients in whom the incidence of neurological side effects could be properly evaluated (Table 7). Of the three patients who received doses of more than 4.5 mg/kg/day (average, 5.8 mg/kg/day), all developed neurotoxic symptoms such as perioral paraesthesias, generalized pruritis, dizziness, and ataxia. In none of the three cases was the initial BUN elevated. The remaining 11 patients each received 4.5 mg/kg/day or less (average, 3.1 mg/kg/day), and only two of this group complained of neurotoxic symptoms. One of these

Table 7. Incidence of Neurotoxicity with Colistin Therapy

Dose, mg/kg/day	Number of cases	Number of cases with initial BUN* elevated	Number of cases with neurotoxicity
>4.5	3	0	3
4.5-3.5	5	1	1
<3.5	6	3	1

*BUN, Blood Urea Nitrogen, mg/100 ml.

patients received only 1.7 mg/kg/day, but had markedly impaired renal function (serum creatinine 5.6). The second received 4.5 mg/kg/day and had a BUN of 27. A third patient complained of paraesthesias during the first day of therapy when he was acutely ill and dehydrated, but after hydration he had no complaints with the same or higher doses.

In five adults and five infants it was possible to evaluate the incidence and severity of nephrotoxicity as reflected by changes in the Blood Urea Nitrogen. These results are presented in Table 8. Five of the ten cases showed a drop in BUN while on colistin, and in only two cases did the BUN rise more than 10 mg/100 ml.

Table 8. Effect of Colistin on Renal Function

Case number	Dose, mg/kg/day	Initial BUN*	Change in BUN*
Adults			
5	4.5	27	+9
21	4.3	25	+2
19	3.1	29	- 4†, +20‡
26	2.2	15	+7
16	2.0	26	- 5
Infants			
12	9.1	16	+10
20	6.0	20	- 6
27	5.0	11	+12
2	4.1	15	- 2
25	2.4	16	- 4

*BUN, Blood Urea Nitrogen, mg/100 ml.
†After 11 days of therapy.
‡After 20 days of therapy.

DISCUSSION

Our data indicate that colistin is an effective agent in the treatment of Pseudomonas urinary tract infections resistant to other antibiotics. Ten of 17 such cases were cured of their Pseudomonas infection, and an additional six showed distinct improvement. Moreover, neurotoxic side effects were rare with doses of 4.5 mg/day or less, and nephrotoxicity was generally limited to a small rise in BUN even in those with impaired renal function. These results would seem to be at least as good as those with polymyxin and without the troublesome toxic side effects frequently seen with the latter drug. Pulaski and Rosenburg [5], for example, reported on 20 cases of urinary tract infections treated with polymyxin. Of the eight cases due to Pseudomonas, five were cured and three improved. Neurotoxic symptoms in that series occurred in "practically every instance," the mean dose used being 2.5 mg/kg/day.

Hopes that colistin would be more effective than polymyxin in treating Pseudomonas infections other than those involving the urinary tract are not supported by our data. The failure of colistin in the treatment of Pseudomonas infections elsewhere in the body is exemplified by case 21. The patient's urinary tract infection was rapidly cleared by colistin while blood cultures continued to be positive because of an infected gall bladder and heart valve. Patients with pulmonary infections and septicemia likewise generally had a questionable response to colistin therapy. These findings are consistent with those of others such as McCabe [6] who reported a failure of Pseudomonas pneumonia to respond to colistin, with fatal meningitis developing during therapy.

The reason for the favorable response in urinary infections is undoubtedly due to the high concentrations of the drug attained in the urine with usual doses of colistin. Blood levels of colistin on the other hand are low even with the higher doses of colistin. Those levels reported in the present study ranged from 3.3 to 6.7 μg/ml with 16- or 33-mg doses, and are somewhat higher than those previously reported by others, a difference explainable by our study of ill patients and their likelihood of having some impairment of renal function. However, even these serum levels of colistin are low in relationship to the minimal inhibitory concentrations required for many Pseudomonas isolates. Using colistimethate sodium standard we found that from 2 to 10.7 μg/ml (mean, 3.9 μg/ml) were needed to inhibit clinical isolates. Earlier reports which showed much lower inhibitory concentrations [7] were undoubtedly based on the use of colistin sulfate standard and a relatively small inoculum.

Our data on the relative activity of the two forms of colistin against Pseudomonas isolates support those of Schwartz [8], who also found the colistimethate to be less active than the sulfate with a number of other bacterial species. With Pseudomonas we found the colistimethate to be ½–⅕ as potent as the sulfate when they were compared as "base equivalents." The latter term indicates that sufficient concentrations of the two forms have been prepared so as to produce zones of inhibition equal to that with a given concentration of colistin base, using the cylinder plate method and Bordetella bronchiseptica ATCC 4617. Because of differences in diffusion in agar, drug deterioration, and relative activity against other organisms, equal inhibition zone sizes do not necessarily imply equal activity. The colistimethate is also increasingly less effective than the sulfate against large inocula, such as would be encountered in clinical infections [8]. For these reasons it would seem likely that clinically useful information is only provided by employing colistimethate sodium standard when testing clinical isolates for susceptibility to colistin.

The impressively low toxicity of colistin and the results of susceptibility tests of Pseudomonas strains isolated from patients in this study indicate that in patients with normal renal function daily doses of 5 mg/kg in adults and 7-10 mg/kg in children are feasible and desirable for treating severe infections. Our favorable results even with generally lower doses in the therapy of resistant Pseudomonas infections involving the urinary tract lead us to consider colistin the drug of choice for this clinical problem.

SUMMARY AND CONCLUSIONS

The results of colistin treatment of 28 cases of severe Pseudomonas infections are presented. Twenty-four had strains of Pseudomonas resistant to most or all of the usual antimicrobial agents. Seventeen had urinary infections, and of these ten were cured, six distinctly improved, and there was one treatment failure. Of the remaining eleven cases, five had pulmonary infections, four septicemia, and two endocarditis. Only two of these eleven were cured. Failure to obtain good results in other than urinary tract infections was attributed to the relative resistance of these Pseudomonas strains to the colistimethate form of colistin in comparison with serum levels of the antibiotic. Neurotoxicity was rare, occurring in only two of eleven cases receiving 4.5 mg/kg/day or less of the drug. Of ten cases evaluated for changes in renal function, four had a drop in BUN and in only two was there a rise in BUN greater than 10 mg/100 ml.

Colistin is thought to be at least as effective as polymyxin B in Pseudomonas infections involving the urinary tract resistant to other antibiotics and, because

of the lower incidence of toxic side effects with colistin, the latter antibiotic is considered to be the treatment of choice. For treatment of Pseudomonas infections elsewhere in the body high daily doses of colistin, 5 mg/kg in adults and 7-10 mg/kg in children, are probably indicated for patients with normal renal function.

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DISCUSSION

DR. CHAWN (Detroit): We have performed some colistin sensitivity determinations with Pseudomonas strains by the dilution method. Of eight strains, one was sensitive to 25 μg, one to 50 μg and six to 100 μg or more. Thus the in vitro studies did not look encouraging to me.

DR. ROBERTS: I have no comment on that except to say that this is unusual, and, in fact, unknown in the published data and certainly did not occur in the 30 isolates which we tested carefully by the tube-dilution method. I would make one point in this regard, though, and that is that it sometimes is quite difficult to get a pure culture in isolates from patients where Pseudomonas is involved and, in previous studies where resistance has been shown to polymyxin-like drugs by Pseudomonas, careful restudy has occasionally or has usually shown a contaminating organism. I'm sure this wasn't true in your case, but it is worthy of thought.

DR. ROSS: I might say parenthetically that our own experience with colistin corroborates Dr. Roberts' experience. We determined the sensitivities of 24 strains of Pseudomonas last year and in no instance did we encounter any strains resistant to colistin.

DR. SCHWARTZ: Reports from the literature from Japan, France, Italy, and this country indicate that the majority of Pseudomonas strains are very sensitive to colistin. I would corroborate Dr. Roberts' statement that it is not unusual to mistake Proteus for Pseudomonas. Proteus is not sensitive to colistin and I think, if you examine these strains very carefully, I wouldn't be suprised at all that some of these are actually Proteus strains, rather than Pseudomona.

DR. RUTENBERG: There are several points I would like to make and one of them, in the management of urinary tract infection, particularly the chronic type, concerns failures which are usually recorded as failures, and are not due to failure of antibiotic therapy. They are usually due to the presence of anatomical or pathological factors in the urinary tract, such as hydronephrosis, obstructing prostate, presence of foreign body, calculi in the bladder or in the pelvis of the kidney, or other static factors which do not render that particular urinary tract susceptible to eradication of the responsible bacteria. And whenever relapse or continuation of infection is noted, it is invariably due to these factors and elimination or proper management of the urinary tract in such cases will facilitate the cure. Also I would like to raise an objection to the term "improved" in evaluating the response of the patient in urinary tract infection. Either the patient is cured or he is not cured and if there is temporary improvement with a relapse which requires further therapy or a long convalescence you really aren't accomplishing very much. The results with colistin and the side effects with this drug do not seem to be very different from those which have been reported with polymyxin. Chemically, both drugs are very similar, the hydrolysis products of these compounds are quite similar, and there is also similarity in their antibacterial spectra and in the side effects. If polymyxin is used for short periods of time, generally 5-10 days since longer periods of therapy are really not necessary, and if the dosage is 1.5 mg/kg body-weight and the patient has good renal function, I think that the therapeutic results are just as good as those which have been reported with colistin. Furthermore, the side effects with polymyxin, provided the dosage and duration of therapy are limited, are not particularly serious. Patients occasionally will develop some paraesthesias around the lip, tingling of the fingers, increase in the BUN, and some changes in the urinary sediment, but if the patient is followed carefully, as obviously the patients in Dr. Hartman's series were, the drug can be given perfectly safely and with just as good results as have been recorded here today.

DR. SCHWARTZ: First of all, colistin is not the same as polymyxin in spite of the fact that they are both polypeptide antibiotics. The generic name for this material is colistin. There is a salt that is called colistin sulfate. Another compound, colistin methate sulfate or colistin methene sodium, is not a salt of this base, but is a new derivative which has different activity than colistin sulfate and it is the only salt that is recommended for intramuscular use.

DR. ROSS: Our own experience with colistin in children does not coincide with yours insofar as toxic equivalence to polymyxin. We tested a series of children using comparable parenteral doses of both colistin and polymyxin and found that there was a rather significant difference, at least in the pediatric age group, between the two drugs. The only discernible evidence of toxicity to colistin was an occasional slight and transitory elevation of BUN, primarily in infants under 6–7 months of age, which reverted to normal after discontinuation of the drug. However, with polymyxin the incidence of side reactions was considerably more abundant. In regard to Dr. Roberts' dosage recommendation in adults, we have used dosages of 7.5 mg/kg/day intramuscularly and have obtained moderately good blood levels in the pediatric age group. I do not believe that the latter dose is an excessive one and it could perhaps even be exceeded without untoward sequellae.

DR. KIRBY: I worked with Dr. Roberts on this study and since the comparison has come up I would just like to make one comparative statement. About six years ago we spent about 18 months working on polymyxin parenterally and I can say that it was very difficult to do this. We had a great deal of difficulty getting any cooperation in treating the patients and difficulty in showing results. Eventually we more or less abandoned it and have rarely, if ever, used polymyxin parenterally since. The experience with the colistin has been quite different. It is certainly marginal in the severe endocarditis cases. It is certainly not marginal in urinary tract infections and during the past year in the Seattle area, in the cases we have studied and selected, the results have been sufficiently impressive that we have had difficulty in having adequate amounts of the drug on hand. I think there is a definite difference in the usefulness and tolerability between colistin and polymyxin.

DR. RUTENBERG: I have not had any experience with colistin, so I cannot give a comparative assessment of the two drugs. However, polymyxin has been used in Boston at the various hospitals where I am associated for 5 or 6 years and although polymyxin has rarely been used in severe systemic Pseudomonas infections, when used, the drug has been satisfactory, and there has been no side effect which was serious enough to warrant its discontinuance. In the early days of polymyxin there was considerable discussion about the various forms of the drug that were available. There were polymyxins A, C, D, and E, forms which varied slightly in their formulation. Some were said to be more toxic than others and finally polymyxin B was recommended as being the most satisfactory. This seems to be a similar situation with the various derivatives of colistin which are under investigation at the moment.

DR. LONG: I will support Dr. Rutenberg's statement relative to polymyxin. Eight years ago he and I treated fourteen patients suffering from Pseudomonas meningitis with polymyxin B. All recovered, and since that time I have seen about a half a dozen more, all treated with polymyxin B. All have recovered.

CLINICAL FINDINGS AND RESULTS OF A THERAPEUTIC REGIMEN IN ACUTE FRIEDLANDER'S PNEUMONIA

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INTRODUCTION

A mortality of 70% in 13 patients with acute Friedlander's pneumonia was reported from this hospital in 1956 [1]. The urgency of early diagnosis and the need for prompt institution of appropriate therapy were emphasized by the occurrence of all the deaths within 48 hr of admission. This series of patients also illustrated the ineffectiveness of penicillin therapy, which was usually employed in the mistaken belief that pneumococcal pneumonia was being treated.

Since that time all patients with suspected or established Friedlander's pneumonia or alcoholic patients with leukocyte counts of 5000/mm^3 or less have been treated with streptomycin and either chloramphenicol or tetracycline. Some of the patients thus treated did not have Friedlander's pneumonia, but this chemotherapy was quite adequate for pneumococcal infections and the benefit obtained in those with Friedlander's pneumonia justified the procedure. This paper is a report of the results with ten consecutive patients suffering from Friedlander's pneumonia who were treated with this regimen since the previous report.

CLINICAL DATA AND THERAPY

The clinical findings and therapeutic data are summarized in Table 1. Males predominated in this series as in the previous one; there were eight, all of whom were alcoholics; in fact three were either acutely intoxicated or in delirium tremens at the time of admission. The patients ranged in age from 31 to 68 years with an average of 46 years. Only two patients had the bloody, mucoid, tenacious sputum characteristically described in this condition. While five had leukocyte counts of 10,000/mm^3 or less, only two patients had frank leukopenia. As in other series [2] the right upper lobe was involved most frequently, but then only in four patients. Bulging of the short fissure, a radiologic sign suggesting Friedlander's pneumonia [3], was present in only two of the ten patients and was therefore decreased in diagnostic importance.

The heterogeneity of the clinical findings indicates that one cannot put a great deal of emphasis on them in diagnosing an individual patient. On the other hand, tentative identification of the etiologic agent was made from the sputum smear on admission in six patients indicating that this simple procedure, together with a high index of suspicion of male alcoholics with relative leukopenia, is the most important factor in early diagnosis. One cannot wait for the results of sputum

Table 1. Summary of Pertinent Data in Ten Cases of Friedlander's Pneumonia

Patient, age, sex	Duration of illness	Condition on admission	Coexisting problems	Sputum	Cultures—time from admission when identified		White blood count	Chest roentgen-ogram	Antibiotic therapy* and hospital course
					Blood	Sputum			
1. 41, M	4	Moderately ill	Alcoholism	Mucoid	F 24 hr	F 24 hr	3,800	RLL	CHL, STR and Ster. Discharged 5 weeks later with residual cavitation.
2. 43, M	7	Moderately ill	Alcoholism	Whitish	Neg × 2	F 2nd day	21,200	RLL	T and STR, 9 days. Then CHL and STR. Complete recovery 5 weeks.
3. 43, M	5	Moderately ill	Epilepsy, recent P.C. † pneumonia	None	Neg × 2	F 24 hr	14,650	LLL	T and STR, 2 weeks. No residual.
4. 56, M	6	Severely ill	Alcoholism	Thick yellow	Neg × 2	F 3rd day	11,500	LUL	CHL and STR, 3 weeks. Then retreatment for bronchiectasis and fibrosis.
5. 31, M	1	Severely ill, DT's	Alcoholism, epilepsy	Thick yellow	Not done	F 48 hr	6,100	RUL	Coexisting active TBC. CHL and STR initially, then INH and PAS. Contracted lobe.
6. 33, M	1	Severely ill, drunk	DT's	Bloody	Neg × 2	F 24 hr	7,300	RUL	E, 4 days, then T and STR, 25 days. No residual.
7. 54, F	10	Severely ill	Heart failure	White mucoid	Not done	F 48 hr	3,000	LLL RML	T, 0.5 and STR, 0.5 (2 doses each). Died 10 hr after admission.
8. 41, M	?	Severely ill	Acute and chronic alcoholic	Purulent	F 48 hr	F 48 hr	10,000	RUL RML RLL	CHL, 1.0 g and STR, 0.5 g (1 dose each). Died 12 hr after admission.
9. 68, F	4	Severely ill	Bronchiectasis	Bloody	Not done	F 2nd day	16,000	RLL LLL	Shock, CHL, STR, and Ster. Died 4 days after admission of asphyxia.
10. 53, M	?	Severely ill, DT's	Alcoholism, DT's	Purulent	F 3rd day	F 72 hr	8,900	RUL	CHL, STR, and Ster. Died of asphyxia 13 days after admission.

*CHL, Chloramphenicol; T, Tetracycline; E, Erythromycin; STR, Streptomycin; STER, Steroids; TBC, Tuberculosis; INH, isoniazid; PAS, para-aminosalicylic.
†P. C. pneumonococcal.

and blood cultures; definitive identification of the microorganism required at least 24 and occasionally as long as 96 hr. It is important in this regard to note that two of the patients in this series died within 12 hr of admission and all nine deaths in the previous series occurred within 48 hr of admission.

Six patients were treated with chloramphenicol and streptomycin while three received tetracycline and streptomycin. One received 2 g/day of erythromycin until the results of sputum culture were obtained, after which tetracycline and streptomycin were employed.

During the acute phase of the patient's illness 1 g of chloramphenicol or 0.5 g of tetracycline was administered by intravenous infusion over a 30- to 45-min period every 6 hr. Simultaneously, 0.5 g of streptomycin was given intramuscularly. After clinical improvement the broad-spectrum antibiotic was given orally; at this time the dose of chloramphenicol was usually reduced to 2 g/day.

Adjunctive measures, particularly bronchoscopy, are often life-saving. This procedure was required in five patients (No. 1, 4, 5, 9, and 10). Its importance was brought out sharply by the outcome in patients 9 and 10. Both were scheduled for emergency bronchoscopy but died just before undergoing the procedure. Autopsy in both instances revealed that the contents of a large abscess had been aspirated into the bronchial tree causing death by asphyxiation. It has been our practice to perform tracheostomy whenever the patient is in distress due to copious or very tenacious sputum, or when weakness or delirium tremens prevents an effective cough. The ease with which bronchoscopy or tracheal toilet can be carried out through a tracheostomy is a decided help in management.

This regimen of streptomycin plus chloramphenicol or tetracycline and vigorous use of adjunctive measures has, in our hands, reduced the mortality of this condition from 70 to 40%. Three of the six survivors in the present series had residual pulmonary damage. Patient 1 refused lobectomy for residual cavitation in the right lower lobe. Bronchiectasis and fibrosis of the involved left upper lobe resulted from the condition in patient 4. Active tuberculosis coexisted with Friedlander's infection in patient 5 making it impossible to say which infection was responsible for the resulting contracted lobe.

DISCUSSION

Acute pneumonia due to the Friedlander bacillus often develops so explosively that one or several lobes of the lung may be entirely destroyed in a matter of hours. Host factors, particularly the decreased resistance brought about by alcoholism, undoubtedly contribute to the seriousness of the infection. Strains of Klebsiella pneumoniae isolated in our laboratory have generally been inhibited by chloramphenicol or the tetracyclines in concentrations of the order of 3-5 μg/ml. Unlike our experience in the previous series [1], none of the organisms isolated from the present group of patients was resistant to either streptomycin or broad-spectrum antibiotics.

The reduction in mortality achieved by this regimen is encouraging. Antimicrobial therapy appears to have been adequate and none of the four deaths are attributable to failure of the antibiotics to influence the organism. Two died 10 and 12 hr after admission, before therapy could be effective, while the other two deaths occurred from asphyxiation at a time when the antibiotics appeared to be containing the infection. The first two fatalities emphasize the limitations of antibiotics while the second pair emphasizes the need for vigorous employment of adjunctive measures.

One wonders whether the first two patients might represent the irreducible minimum mortality attainable under the conditions imposed by a general hospital population, one characterized by alcoholism, debility, and delay in seeking medical attention.

As emphasized in the previous report, one should strongly suspect Friedlander's pneumonia in alcoholic male patients with relative leukopenia. Pending definitive identification of the causative microorganism, the patient should be treated as though he had Friedlander's infection since pneumococcal pneumonia, if present instead, will also be adequately treated by this regimen. The importance of adjunctive measures such as oxygen, bronchoscopy, and tracheostomy cannot be overemphasized.

SUMMARY

Ten consecutive patients with Friedlander's pneumonia were treated with streptomycin and either chloramphenicol or a tetracycline. Six patients recovered. None of the four deaths was considered to be due to the failure of the antibiotic to affect the organism. This series of patients emphasizes the importance of initiating treatment on presumptive grounds in alcoholic male pneumonia patients who have relative leukopenia, and further emphasizes the importance of adjunctive measures, especially bronchoscopy, in the management of this condition.

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CLINICAL USE OF VANCOMYCIN

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INTRODUCTION

Staphylococcal infections remain a serious medical problem. Staphylococci have demonstrated a remarkable ability to develop resistance to antibiotics. Many antibiotics which were formerly useful in the management of these infections are now virtually valueless for this purpose. Since vancomycin* has become available it is apparent that it offers unusual promise in the management of staphylococcal disease. This paper is concerned with the use of this agent in a series of 85 patients.

MATERIALS AND METHODS

The antibiograms of 100 isolates of Staphylococcus aureus obtained between March and May of 1959 were studied by the plate-dilution sensitivity technique to serve as an index of the general sensitivity pattern existing in these Medical Centers. Serial twofold dilutions of the various antibiotics were incorporated in heart infusion agar (BBL) plates. Kanamycin, neomycin, and streptomycin were dissolved in Sorensen's phosphate buffer at pH 8.0. All other antibiotics were dissolved in sterile distilled water. Ten strains were tested per plate using a standard loopful of a 24-hr heart infusion broth (BBL) culture as inoculum. For 50 of the strains the inoculum consisted of undiluted broth cultures; for the other 50 a 10^{-2} dilution was used.

The clinical records of 85 patients who received vancomycin during the past two years have been reviewed (types of infection treated are noted in Table 1). The hospital population receiving this drug consisted of inpatients at the Veterans Administration and UCLA Medical Centers in Los Angeles. For the most part these were acutely ill patients, either old, or debilitated, or both and the use of vancomycin was restricted to severe antibiotic-resistant infections or infections which had failed to respond to previous antibiotic therapy. Eighty-two of the patients were male and three were female. Ages ranged from 17 to 89 years. Duration of treatment varied from one to 42 days. Cultural documentation of the type of infection was obtained in virtually all cases. These cultures were processed in the clinical bacteriology laboratory.

Vancomycin was administered orally, intravenously, or by both routes. For oral administration the parenteral preparation was merely dissolved in water and then given to the patient. Two methods were employed for intravenous administration. Fifty-nine patients received the drug in divided dosage over a 30- to 90-min period. Twenty-six patients received the drug by constant drip over a 24-hr period. Daily dosage given intravenously ranged from 1 g in those

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*Supplied through the courtesy of Dr. R. S. Griffith, Eli Lilly and Company, Indianapolis.

Table 1. Staphylococcal Infections Treated

Infection	Cases
1. Septicemia and Endocarditis	33 (38.8%)
a. Staphylococcal	31
b. Pneumococcal	1
c. Micrococcal	1
2. Pneumonia	20 (23.5%)
3. Enterocolitis	10 (11.8%)
4. Wound Infections	12 (14.1%)
a. Abdominal	
1) Peritonitis	3
2) Subphrenic and hepatic abscesses	3
3) Infected aortic graft	1
b. Osteomyelitis	5
5. Miscellaneous	10 (11.8%)
Total	85 (100%)

Table 2. Antibiotic Resistance in 100 Isolates of *S. aureus*

Antibiotic	Concentration, μg/ml	Resistant strains, %
Novobiocin	>20	4
Kanamycin	>62.5	37
Neomycin	>20 (bactericidal end point)	33
Penicillin G	>12	85
Bacitracin	> 5	32
Chloramphenicol	>25	35
Erythromycin	>10	6
Tetracycline	> 5	87
Streptomycin	>20	63
Vancomycin	>10	0

patients with renal impairment to 3 g. The amount of drug given orally ranged from 3 to 8 g daily in most cases, however, one patient received 12 g daily.

RESULTS

Results of plate-dilution sensitivity tests are given in Table 2. A significant percentage of our strains of *S. aureus* are resistant to penicillin G, streptomycin, tetracycline, chloramphenicol, kanamycin, neomycin and bacitracin. None of the strains were resistant to vancomycin.

Clinical material. Thirty-three patients were treated for septicemia and endocarditis and of these 18 or 54.5% expired (Table 3). Twelve of the 18 deaths occurred under circumstances unrelated to infection, such as acute myocardial infarction, massive pulmonary embolization, hepatic coma, acute blastic leukemia, and ruptured aortic aneurysm. Three patients developed superinfections, two of whom died of Pseudomonas septicemia and Klebsiella pneumonia, respectively. The third patient developed a Gram-negative septicemia which responded to therapy. Only four of the 18 deaths were due to staphylococcal infection. Three of these patients received the drug for 24 hr or less. One patient, however, was treated intravenously for 5 days with 3 g daily with good clinical response. Vancomycin was then discontinued and the patient was placed on erythromycin and chloramphenicol, to which the organism was sensitive. However, 3 days later blood cultures were again positive and the patient expired due to sepsis.

Table 3. Mortality and Superinfections Occurring in Patients with Various Infections

Infection	Number of patients	Mortality				Superinfections
		Staphylococcal infection	Superinfection	Unrelated to sepsis	Total	
Septicemia and endocarditis	33	4	2	12	18 (55%)	3 (9%)
Pneumonia	20	7	2	4	13 (65%)	3 (15%)
Enterocolitis	10	0	0	3	3 (30%)	1 (10%)
Wound infections	12	1	3	1	5 (41%)	4 (25%)
Miscellaneous	10	0	1	3	4 (40%)	1 (10%)
Total	85	12	8	23	43 (51%)	12 (14%)

Following commissurotomy, one patient with rheumatic heart disease and mitral stenosis developed staphylococcal endocarditis. Positive blood cultures continued during intravenous vancomycin therapy despite the fact that the organisms were still sensitive to vancomycin. Following the addition of kanamycin, the blood cultures became sterile but the patient did not become afebrile until vancomycin was discontinued. He is the only patient in the series who did not respond although he received an adequate course of therapy.

Patients with staphylococcal pneumonia were treated with vancomycin with a mortality rate of 65% (Table 3). Seven of the 13 patients who expired did so as a consequence of staphylococcal infection. Two of these were receiving adrenal corticosteroids concurrently. Two patients received less than 48 hr of vancomycin therapy. In another instance, after 4 days of combined vancomycin and kanamycin therapy vancomycin was discontinued. Sputum culture the following day was positive for S. aureus resistant to kanamycin. Vancomycin was renewed but the patient expired within 24 hr. One patient with chronic pulmonary disease expired of respiratory failure associated with staphylococcal infection. Two deaths occurred as a result of aspiration; in one there was distinct clinical improvement prior to aspiration. Three superinfections occurred; two patients with Pseudomonas septicemia died and one Klebsiella pneumonia recovered.

Ten patients with staphylococcal enterocolitis were treated (Table 3). Three deaths occurred in this group, none of which could be directly related to staphylococcal infection. Post-mortem examinations in all three failed to reveal any cultural evidence of S. aureus. One patient died of peritonitis, exudate from which failed to yield any growth aerobically. One patient died of an acute myocardial infarction and the third death was due to hepatic coma. Only one patient developed a superinfection, a Pseudomonas pyelonephritis. It should be pointed out that this patient had also received intravenous vancomycin. One patient who responded to oral vancomycin alone, three weeks later developed a staphylococcal septicemia that responded to vancomycin intravenously; unfortunately he subsequently died of acute myeloblastic leukemia. Five of these ten patients received oral therapy alone and in this group the response was as good as in those treated with combined oral and intravenous therapy. There were no deaths either due to or related to sepsis.

The fourth group of patients who received vancomycin therapy were those with infected wounds or related infections (Table 3). Three of these patients had peritonitis and two of them died, one of intra-abdominal staphylococcal abscesses and the other of Escherichia coli and Pseudomonas septicemia. One patient, following lumbar sympathectomy and iliac endarterectomy, developed a staphylococcal aortitis with subsequent perforation. Although this was repaired, the patient developed a Klebsiella superinfection and expired. Of three patients with subphrenic and/or intrahepatic abscesses, only one expired; his death was due to causes other than sepsis. Of the patients treated for osteomyelitis, only one died and this was due to a Gram-negative superinfection. Three patients had closure of the superficial wounds but there has been insufficient follow-up to determine whether cure has been effected. One patient in whom surgical removal of a sequestrum was performed in conjunction with parenteral vancomycin therapy was cured.

Ten patients are in the miscellaneous category (Table 3). This group included one case of pansinusitis, two of endophthalmitis, and one corneal abscess, all due to S. aureus. The staphylococcal infection was controlled in all cases but two patients lost the affected eye and one died of Pseudomonas septicemia. One case of chronic pyelonephritis due to S. aureus had negative urine cultures for approximately six weeks following cessation of therapy. The remaining five patients were treated with vancomycin under the mistaken impression that S. aureus infection was involved. One patient had tuberculous meningitis and the remainder lymphoma, melanoma, or leukemia. In these patients, the subsequent course established that the fever could be attributed only to the primary disease.

Therapeutic results in those patients receiving vancomycin by intermittent intravenous administration have been compared with patients receiving the drug by constant intravenous infusion. In this analysis we have eliminated five cases of enterocolitis since they also received oral therapy which alone could have been curative, five patients who actually did not have an infection, and several other patients who could not be evaluated because they received nonstandard dosage. of the remaining patients, 21 received vancomycin by constant intravenous infusion and of these nine were considered treatment failures. Among the 47 patients receiving the drug intermittently only ten were treatment failures. There was no difference in the case material between the two groups treated which might account for the difference in response.

Side effects resulting from vancomycin administration occurred in 50 of the 85 patients treated (Table 4). No patient who received oral vancomycin alone had any side effects. One patient who received 12 g orally as well as 2 g intravenously had transient elevation of his serum creatinine.

The commonest complication was thrombophlebitis. This occurred in 38 patients and in 32 was the only undesirable side effect. In no instance were we required to discontinue vancomycin therapy because of thrombophlebitis. In contrast to the belief of Geraci and Heilman [1] and supporting the findings of Dutton and Elmes [2] and Kirby, et al. [3], we found a significant difference in the incidence of thrombophlebitis developing in patients receiving vancomycin by constant intravenous infusion (16 patients or 62% of patients treated in this manner) as opposed to those receiving divided doses administered over a 30- to 90-min period (22 patients or 37%).

Table 4. Side Reactions in 85 Patients

Side reactions	Number of cases
Thrombophlebitis	38 (45%)
Thrombophlebitis only	32
Nephrotoxicity	11 (13%)
Nephrotoxicity only	8
Ototoxicity	3 (3.5%)
Ototoxicity only	0
Possible drug fever	4 (4%)
Drug fever only	1
Possible rash	1 (1%)
Rash only	0
Leukopenia	2 (2%)
Leukopenia only	1

Nephrotoxicity was determined by following serum creatinine levels. Elevations of at least 0.5 mg/100 ml, which were otherwise unexplained, were considered to represent nephrotoxicity. Such elevations occurred in 11 patients (13%). Eight patients had no prior history or evidence of renal impairment. Of these patients, one expired of superinfection and there was, therefore, no opportunity to determine reversibility. Serum creatinine returned to pretreatment levels in three to four weeks in five patients and in the remaining two were significantly lowered one to two weeks after discontinuing vancomycin. Unfortunately, there was no further laboratory follow-up in the latter two patients. Of three patients with prior azotemia, the creatinine in one patient returned to pretreatment levels within two weeks of completion of therapy and the remaining two patients expired due to renal failure. The case reports on the latter two patients are as follows.

Case 1: A.W., a 65-year-old caucasian male, was transferred to the Orthopedic service in December, 1959 because of a fracture of the left humerus and wound infection. Five months after admission the humeral head was resected because of necrosis. One month later he devoted a sudden temperature spike to 103 C and became hypotensive. Serial blood cultures taken at that time were positive for S. aureus, sensitive to kanamycin and chloramphenicol. At the time the patient was started on kanamycin the serum creatinine was 1.3 mg/100 ml. After five days of kanamycin serum creatinine had risen to 3.1 mg/100 ml; vancomycin, 2 g intravenously, was then substituted. Within 24 hr temperature was normal and the patient clinically improved. However, after three days of vancomycin, the patient became anuric and serum creatinine was 10.5 mg/100 ml. Vancomycin was discontinued by the patient expired three days later due to renal failure.

Case 2: J.D-L, a 52-year-old caucasian male, was hospitalized for arteriolar nephrosclerosis and chronic pyelonephritis. During his hospitalization serum creatinines ranged from 6-8 mg/100 ml. He developed a staphylococcal pneumonia during hospitalization. Vancomycin, 2 g intravenously, was begun at a time when the serum creatinine was 8.0 mg/100 ml. After 24 hr of therapy the serum creatinine was 14.0 mg/100 ml. The patient expired of renal failure.

Audiometric examinations were not performed routinely but all patients were observed clinically for ototoxicity. Objective and/or subjective evidence of auditory nerve damage appeared in three patients (3.5%). Two of the three had renal impairment. In spite of this only one had audiometric evidence of minimal high tone hearing loss. The other had temporary tinnitus without audiometric changes. The third patient received vancomycin and kanamycin concurrently. Four days after cessation of vancomycin therapy, while still receiving kanamycin, audiometric findings of impaired high tone function appeared. He was left with minimal residual impairment.

Drug fever was suspected in four patients but three had associated thrombophlebitis. Both the febrile state and thrombophlebitis improved after vancomycin was stopped. The fourth patient developed chills and fever on the last 2 of his 24 days of therapy.

A skin rash appeared in only one instance. This was a macular nonpruritic rash limited to the thorax.

Table 5. Blood Count Record, Case 1

Date	WBC	PMN's	Bands	EOS	Lymphocytes	Monocytes
10-29	15.3	82	1		15	
11-4	5.9	73			15	
11-7	6.6	70	2			
11-16	7.4					
11-19	5.2					
11-23	4.9	65	1	3	21	
11-24	5.4	71		3	21	
12-5	2.3	18	8		35	38
12-7	2.0	16	4	1	63	16
12-9	3.7	44	1	1	35	18
12-11	4.2	48	2	1	38	8
12-14	3.9	53			33	9

Table 6. Blood Count Record, Case 2

Date	WBC	PMN's	Bands	EOS	Lymphocytes	Monocytes
12-10	5.6	69	1	5	14	9
12-14	7.2	35	2	16	35	11
12-15	7.5	35	12	14	21	12
12-16	14.2	72	10	5	11	4
12-21	3.1	24	12	7	38	13
12-22	7.0	29	9	8	36	9
12-23	5.7	39	7	9	32	12
12-24	14.8	64	9	2	17	6
12-28	10.4	58	5	4	27	5
12-31	10.2	62	1	2	25	6

Two patients developed neutropenia while receiving vancomycin. Bone marrow aspiration was done in one patient and confirmed arrest in the myelocytic series. In neither instance was there evidence of thrombocytopenia or anemia related to vancomycin therapy and in both the neutrophile count had returned to normal within 10 days after vancomycin was stopped (Tables 5, 6). Case reports on these two patients are as follows:

Case 1: S.Y.C., a 29-year-old oriental male, was hospitalized after being involved in an automobile accident in which he sustained multiple injuries including a fracture of the right patella. The patellar fracture was reduced by wiring and the patient did well until two weeks later. At this time he became febrile but an exhaustive search for the etiology of the fever was nonrevealing. Twenty-seven days after the open reduction there was evidence of a wound infection at the operative site which cultured S. aureus. Blood cultures at this time were positive for the same organisms. Two days later the patient was started on intravenous vancomycin, 2 g daily, and in 48 hr was afebrile. After twenty-three days of vancomycin therapy a blood count showed granulocytopenia (Table 5) and the drug was discontinued. On the following day bone marrow aspiration revealed maturation arrest in the granulocytic series. Within 4 days the white blood cell count had returned to normal limits and the patient continued an uneventful course for the remainder of his hospitalization. This patient had no evidence of renal impairment at any time and there was no history of exposure to any bone marrow depressants.

Case 2: H.B., a 32-year-old caucasian female, had a cholecystectomy and following surgery she received a unit of mismatched blood. Subsequently, she went into renal shutdown and was then transferred to the UCLA Medical Center where she was treated with the artificial kidney and peritoneal dialysis with reestablishment of renal function. These events were complicated by the development of a subhepatic abscess which was drained surgically and cultured S. aureus. At the time of discharge she was afebrile but had anemia and persistent wound drainage. Serum creatinine was normal; the only evidence of renal impairment was her inability to concentrate above a specific gravity of 1.015 following vasopressin. Eight days later, due to right upper quadrant pain and low grade fever, she was readmitted. S. aureus again was cultured from the wound drainage and she was given a course of erythromycin and novobiocin (2 g daily of each). This was discontinued after 8 days because of fever and rash. The patient was started on vancomycin, 1.5 g daily, the following day. After 3 days of vancomycin treatment the patient was re-explored and a sinus tract was excised. Following this procedure, the patient developed temperature spikes preceded by chills. No infection origin could be found and this was felt to be secondary to thrombophlebitis and the possibility of drug fever was entertained. On the 6th postoperative day (9 days after vancomycin was started) the white blood cell count was reported as 3100 (Table 6). Vancomycin was discontinued. She continued to have spiking temperatures for the next 4 days without evidence of infection but then became afebrile without further treatment and within a week was discharged.

DISCUSSION

Results of the clinical data presented indicate that vancomycin is a highly effective agent in treating S. aureus infections. Almost 90% of the infections discussed in this report were life threatening. As shown in Table 2, the S. aureus population in these centers is predominantly resistant to the majority of antibiotics. Vancomycin has been in use for 3 years and we have yet to find a strain showing resistance to over 10 μg/ml.

Mortality rates in staphylococcal sepsis are difficult to compare. Kirby, et al. [2] found, however, that in those series reporting on 25 or more S. aureus infections the mortality rate was over 50%. Wilson and Hamburger [4] at the Cincinnati General Hospital reported a mortality rate of 62% with no difference in those patients with endocarditis as opposed to those who had only septicemia. Geraci, et al. [5], in a series of 28 patients with S. aureus endocarditis treated during a 7-day period, reported a mortality rate of 61%.

Although 18 of 33 patients in this series with septicemia or endocarditis died, only 4 (12%) of these deaths were attributable to S. aureus infections. Of equal importance was the fact that two of these 18 patients died as a result of superinfection with Gram-negative organisms.

The death rate due to sepsis from S. aureus was relatively constant at approximately 10% in all categories discussed, with the exception of the pneumonias. Seven of 13 deaths in this latter group were directly related to S. aureus infection; the overall mortality due to staphylococcal pneumonia was 40%. However, two patients received only 24 hr or less of vancomycin treatment. One patient who died on the third hospital day was receiving adrenal corticosteroids. Another factor influencing the mortality may be severe chronic pulmonary disease which existed in the majority of patients.

Those patients with S. aureus enterocolitis were among the most successfully treated. There were no deaths due to sepsis in those treated orally and these patients responded as well as those receiving supplemental intravenous vancomycin.

Mortality in the total group of 85 patients was 51%. However, mortality due to staphylococcal sepsis was only 15%. These results are comparable to those obtained by Kirby, et al. [3] in a group of 33 S. aureus septicemias.

The better therapeutic results in patients receiving vancomycin intermittently may be related to higher peak blood levels achieved by this technique.

Superinfection was a significant problem. Twelve patients developed superinfection due to Gram-negative bacilli and eight of them died (Table 3). Superinfection has not been encountered as a significant clinical problem in other studies of vancomycin therapy. Our study emphasizes that one should be alert to this possibility, especially if the patient receiving vancomycin shows signs of deterioration after an initially good response.

The majority of side effects ascribed to vancomycin were due to thrombophlebitis. There were no instances of therapy being discontinued because of thrombophlebitis. It is likely that thrombophlebitis would have occurred in some patients as a result of prolonged intravenous therapy involving either a needle or a polyethylene catheter aside from the vancomycin itself. The incidence of thrombophlebitis was reduced when the drug was given intermittently rather than by constant intravenous infusion.

Our series is notable with regard to the incidence of nephrotoxicity. In the patient who received 12 g/day orally as well as 2 g intravenously, it is conceivable that there was some intestinal absorption because of the extensive enterocolitis. The preponderance of older patients in this series may have had some bearing on the relatively high incidence of nephrotoxicity as compared to other series. In the two patients who died of renal failure, we might have avoided major difficulty if we had used smaller dosage.

Only one instance occurred of a patient receiving vancomycin alone and having residual ototoxicity. In the other instance of residual auditory nerve damage the patient had also received kanamycin. It should be pointed out that sequential or concurrent administration of potential ototoxic agents is to be avoided when possible. The association between renal impairment and ototoxicity was again noted in this series and caution must be observed in the presence of renal damage.

In this study there were two instances of neutropenia; one was confirmed by bone marrow aspiration and there was no evidence of renal damage or history of exposure to other potential bone marrow depressants. The second patient who exhibited neutropenia had been treated for acute renal shutdown some three months earlier, had an indolent infection for three months, and had received a course of novobiocin just prior to vancomycin therapy.

The intermittent mode of administration of vancomycin must be considered the method of choice in administering the drug since there is a distinctly lower incidence of thrombophlebitis and there also appears to be a therapeutic advantage. A possible exception to this, however, would be patients with impaired renal function since here intermittent therapy might lead to undesirably high blood levels which could result in further nephrotoxicity and in ototoxicity.

SUMMARY

Eighty-five patients, the majority of whom were suffering from severe Staphylococcus aureus infections, were treated with vancomycin. Forty-three (51%) patients expired but only 13 (15%) of the deaths were due to staphylococcal infection. Mortality due to sepsis was approximately 25%. Twelve superinfections developed, eight of which caused death. Of the side reactions occurring in 50 patients, thrombophlebitis was predominant and nephrotoxicity, ototoxicity, drug fever, skin rash, and two cases of neutropenia were also observed.

These results indicate that vancomycin is remarkably effective in the management of severe S. aureus infection both from the standpoint of increased therapeutic effectiveness and decreased incidence of side effects. It appears desirable to administer the drug intermittently rather than by constant intravenous infusion except in those patients with impaired renal function.

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DISCUSSION

DR. ROMANSKY: I am rather surprised at the high incidence of thrombophlebitis and I think perhaps some of it may be due to the mode of administration, and secondly, I would like to ask if the difference in the mortality rate is related in any way to the group of patients who received the vancocin by the continuous intravenous drip. Our experience has been not as vast in numbers of patients but in 12 patients that we have had only one developed thrombophlebitis and, as some of you perhaps know, in our use of both ristocetin and vancocin we administer the drug in about 100 ml of fluid in roughly 10-15 min. In this way we have been very fortunate in minimizing thrombophlebitis. I would be interested in an answer to both of these questions.

DR. DANGERFIELD: With regard to the high incidence of thrombophlebitis, in the paper that will be submitted for publication we have indicated that we felt this was again high, as you pointed out, but it could probably be related, for instance, to the prolonged therapy that many patients received. I indicated that the range of the duration of therapy ran from 1 to 42 days and I feel that in many of the instances of thrombophlebitis this would have developed due to pure mechanical irritation of having a needle or a polyethylene catheter in place. I was unable to determine, in all of these cases, the exact time level that thrombophlebitis appeared.

Now, with regard to the incidence of mortality rate relative to the mode of administration, I did not break down the figures that way and I couldn't give you an answer.

DR. COTLAR: We certainly have seen thrombophlebitis in almost all the patients receiving intravenous vancomycin. We use a cut-down, preferably in the arm but occassionally, out of necessity, in the leg. As for the time required to develop thrombophlebitis, it generally occurred within 24 hr or less.

DR. DANGERFIELD: At the Veterans Administration Hospital and University Hospital in Los Angeles the preferred mode of administration is by divided dosage, 1/2 dose given in a half an hour and approximately 100 to 200 ml of saline or water 4 times per day. We feel that there is a decrease in the thrombophlebitis using this method.

DR. FREMONT SMITH: (Boston): On this problem of thrombophlebitis, I agree that this is the major problem in the use of the drug. Has anybody the impression that lots of the drug vary in their irritating potential? When the drug first came on the market we found a great incidence of thrombophlebitis. Up to about a year ago, there was a period when we could use it quite with impunity. In the last six months we have had a group of patients all of whom have had thrombophlebitis no matter how we have given the drug. I think our experience is now some 60 or 70 patients. A real problem is presented because, particularly with our patients at the Peter Bent Brigham Hospital, who with their renal failure problems have catheters in practically all their veins and we are really stuck as to how to give the drug in any fashion. Has anybody further thoughts about the lot variations in terms of irritating potential?

DR. ROSS: Our own experience parallels yours in our recent use of vancomycin in the pediatric age group. It is the rare child that gets by without thrombophletitis. In our two most recent cases, one child with acute osteomyelitis and the other with subacute bacterial endocarditis with secondary meningitis, we were forced, after considerable and distressing difficulty with thrombophlebitis, to insert a polyethylene tube into the inferior vena cava and only by that method were we able to maintain continuous intravenous administration of vancomycin for the desired interval, namely, 30 to 40 days.

DR. ROMANSKY: I don't think there is any question but what the earlier lot numbers not only produced a greater incidence of thrombophlebitis but also other side effects such as chills, nausea, etc. In the last eight to ten months we have had rather pure preparations and the side effects have been negligible except for the one instance we have had of thrombophlebitis. I do think, Dr. Smith, that the latest preparations are relatively free of irritation-potential. I still would like to emphasize the importance of two things in the administration of vancomycin and ristocetin. Not only is it important that they be administered rapidly to prevent thrombophlebitis but our experience in the last year emphasized even more the need for administering this total gram dose, in approximately 100 to 150 ml in 10 to 15 min. Now, we have had patients who received these drugs for as long as six weeks or more, receiving two injections a day, one at 12-hr intervals given in 15 to 20 min. But the second reason for this, and perhaps even more important than the phlebitis, is the necessity for getting that high concentration which is so essential in staphylococcal infections since these organisms are not all in the same relative degree of responsiveness to any particular agent and I think this concentration which we get in 2 hr of approximately 20–40 μg is extremely important in reversing the virulence of these infections. At 2 hr we find 20–40 μg and at the end of 12 hr, approximately 5–10 μg/ml by this method. This is actually a more important consideration than the question of the thrombophlebitis.

DR. ROSS: Doctor Kirby, with the advent of Staphcillin what do you think the future of vancomycin is?

DR. KIRBY: We have for some months now been using Staphcillin and vancomycin side by side and I think it is too early to give definitive comparisons of the two drugs. As nearly as we can tell, the response to the two is roughly comparable. They both present problems of administration. The Staphcillin poses problems of having to administer the drug every 4 hr. We also prefer, as does Dr. Romansky, to give vancomycin in 15 or 20 min twice a day. We never give it into a cut-down because I feel sure that the thrombophlebitis is much worse there. With Staphcillin, on the other hand, we have been able, with adjustment of the pH, to give it in a cut-down with less vein irritation but in many patients it is simpler at the present time to give vancomycin, 1 g/12 hr, because it is all over in 15 or 20 min and this solves the problem of administration. This is a better alternative for the patient who has good veins and in whom it can be done carefully. I would say that we cannot state categorically that one is better than the other therapeutically at the present time. There are some instances though, such as in patients with enterococcal endocarditis and general enterococcal infections, where Staphcillin is not effective in reasonable concentrations and in which vancomycin is clearly the drug of choice.

PNEUMOCOCCAL MENINGITIS IN THE ADULT

The Clinical, Therapeutic, and Prognostic Aspects of 43 Patients

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INTRODUCTION

The pneumococcus is uniquely susceptible to penicillin. Despite widespread use, this antibiotic remains bactericidal for the pneumococcus at low concentrations, and no resistant strains have emerged. These unusual characteristics of the pneumococcus have resulted in a uniformly low mortality for pneumococcal infections generally and the disappearance of pneumococcal pneumonia as a therapeutic problem.

Pneumococcal meningitis, however, appears to be an exception to these facts and mortality varies from as low as 7 to as high as 72% in some series [1, 2, 3]. The purpose of this paper is to review our experience with pneumococcal meningitis in 43 adult patients seen during the years 1950-1960 in the George Washington University Medical Service at the District of Columbia General Hospital.

MATERIALS AND METHODS

All of the patients had clinical and spinal fluid findings consistent with purulent meningitis. Pneumococci were cultured from the spinal fluid in all but two instances. The two patients in whom the microorganism could not be recovered had positive smears and characteristic cellular and chemical changes in the spinal fluid in association with positive blood and sputum cultures. The following data were tabulated from the clinical record: age, sex, duration of illness, month of the year, level of consciousness, coexisting conditions, and type of therapy. Laboratory data that were evaluated included blood leukocyte count, spinal fluid smear, cell count, sugar concentration, and the results of blood and spinal fluid cultures.

The usual physical signs of meningitis were present in all patients. The patients were mainly in the older age groups, 31 (72%) being 40 or older and 15 (34%) over sixty. Males predominated by a ratio of 26 to 17. The seasonal incidence of meningitis appeared to parallel that of pneumonia in this hospital; thirty-three of the cases occurred between the beginning of November and the end of April.

DISCUSSION AND RESULTS

In general hospitals, patients with bacterial meningitis are often seen for the first time late in the course of their disease, frequently comatose and with a chronic debilitating illness. In our series the average duration of illness was 2.4 days, 60% of the patients were comatose on admission, and 75% had a complicating major illness. With this patient background the over-all mortality in this series was 65.1%. Weinstein [4] states that poor prognosis in pneumococcal meningitis can be expected with the following: advanced age, delay in instituting therapy, coma, the presence of bacteremia, and complicating diseases. He also states that the prognosis is worse when meningitis follows pneumonia and is of intermediate prognosis with otitis media or similar infections and best when no portal of entry is found.

Figure 1 shows the relationship of mortality to age. In this study, the highest mortality was actually noted in patients between 40 and 59 years of age. This group, however, included most of the chronic alcoholics as well as the patients with otitis media and pneumonia as complicating factors.

Figure 2 correlates mortality with the duration of illness prior to hospitalization. When the delay in hospitalization exceeded three days, mortality rose abruptly to 80%. When the delay was less than three days, even when therapy could be considered adequate, the mortality was still 57%.

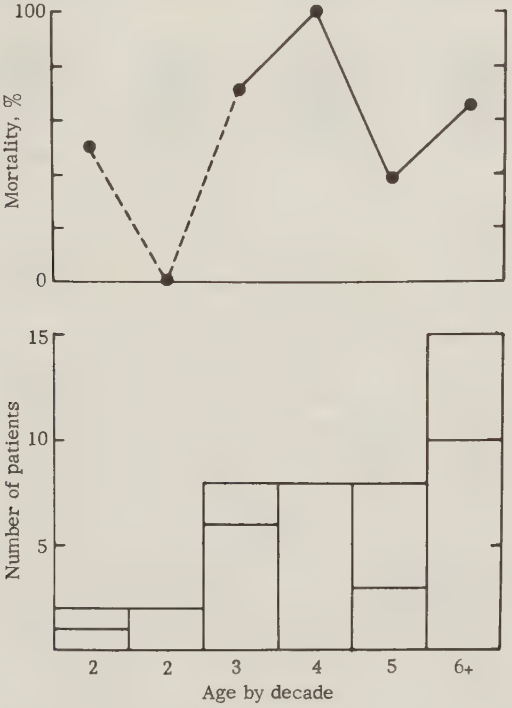

Fig. 1.

It would appear that the level of consciousness is a good clinical index upon which to base prognosis. Mortality rose steadily from 59% in stuporous patients to 77% in those with coma. None of the patients could be considered alert on admission.

Bacteremia was associated with a mortality of 68%. It should be noted here, however, that of the group with bacteremia ten patients had pneumonia and three had otitis media. Pneumonia and otitis media carry a worse prognosis independent of the presence of bacteremia. Of the remaining ten patients who had bacteremia alone, three died, a mortality of 30%.

Complicating diseases exclusive of otitis media and pneumonia were present in 16.7% of patients with a 58% mortality.

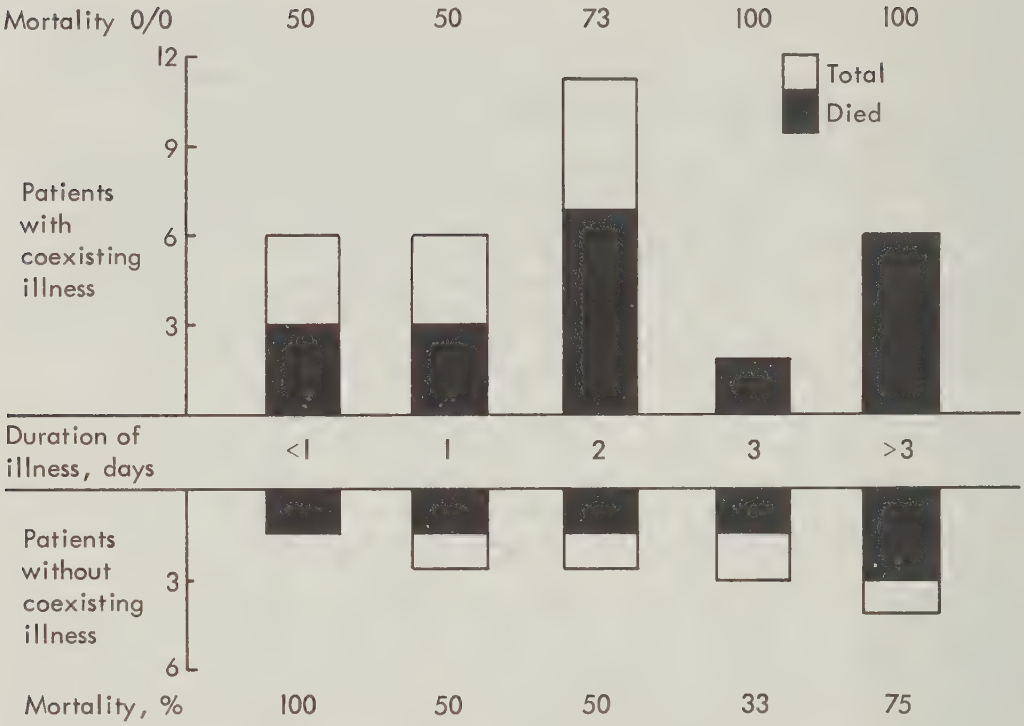

Fig. 2. Pneumococcal meningitis in 43 patients. Duration of illness vs mortality.

The presence or absence of a focus of infection significantly altered the mortality. Sixteen patients had pneumonia with the highest mortality of 87.5%, eight patients had otitis media with an intermediate mortality of 75%, and the lowest mortality of 52.5% occurred in those patients without a demonstrable focus of infection. These findings are consistent with the general statements that pneumonia, sinusitis, or otitis media significantly worsen the prognosis of pneumococcal meningitis.

In those patients in whom the Gram stain of the spinal fluid was reported, it proved to be diagnostic in 97% of the instances. This again demonstrates the value of this simple laboratory tool in providing a definite diagnosis on which to begin specific therapy.

The chemical and cytologic findings in the spinal fluid did not correlate with mortality, nor did the blood leukocyte count.

THERAPY

The results of therapy are detailed in Table 1. Thirty-six of the patients were classified by treatment into one of three major groups: penicillin alone (in varying dosage), penicillin plus one of the tetracyclines, and erythromycin alone. The remaining seven patients represented a wide variety in forms of therapy. There were instances of death before therapy could be evaluated, and one patient died of an anaphylactic reaction to penicillin five hours after admission. Soluble crystalline penicillin was generally administered every two hours by rapid intravenous drip or by the intramuscular route. There is little difference in the results of those patients treated with either 12 or 24 million units of penicillin per day (54% vs 59%). There is also little difference between the patients

Table 1. Results of Therapy in Cases of Pneumococcal Meningitis in the Adult

Therapy	Number of patients	Deaths	Mortality, %
A. Penicillin			
1 million units sol. cryst. iv q2h	13	7	54
2 million units sol. cryst. iv q2h	7	4	59
0.6 million units aq. procaine im qqh	3	1	33
B. Erythromycin			
0.5 g qqh iv	6	3	50
C. Penicillin plus tetracycline group			
1 million units sol. cryst. q2h iv plus 0.5 g chlortetracycline, tetracycline, or oxytetracycline q6h iv	7	6	85

treated with penicillin and those treated with 1 g of erythromycin intravenously every eight hours. The mortality in both groups was approximately 50%. It is of interest that two of the three patients treated with 3.6 million units of procaine-penicillin per day in divided dose survived. None had pneumococcal infections elsewhere in the body and the two survivors had been ill less than a day when therapy was started. These factors probably played an important part in their recovery.

Seven patients received penicillin in combination with one of the tetracyclines (chlortetracycline, two cases; oxytetracycline, three cases; tetracycline, two cases). Six of these patients died, a mortality of 85%. There is nothing in the clinical records to indicate that these patients were in any way more severely ill, or ill for a longer time, than other subjects, suggesting that the tetracyclines actually exerted a deleterious or antagonistic effect on the action of penicillin. Lepper and Dowling [5] reported a similar experience with a mortality of 79% in 14 patients treated with penicillin and aureomycin.

Steroids were employed in only seven patients, four of whom died. Therapy was quite variable, ranging from 50 to 300 mg of hydrocortisone per day. Evaluation of such regimens is obviously impossible.

SUMMARY

We feel that under optimum conditions pneumococcal meningitis should have a mortality no greater than 10%, but that with patients seen at general hospitals the mortality will remain high. The factors that contribute to this higher mortality are: delay in seeking treatment, the frequent association of meningitis with pneumonia and otitis media, and the presence of complicating disease.

The single most important diagnostic procedure is the Gram stain since it provides a simple and rapid means of establishing a definite diagnosis.

The treatment of choice is penicillin but erythromycin in adequate doses is equally effective. The tetracyclines, however, in combination with penicillin, even though both are given in adequate doses, seem to exert an antagonistic effect, as evidenced by a significantly higher mortality.

Infectious diseases, with notable exceptions, are easily treated with the great variety of antibiotics available today. The limiting factors then become early diagnosis and prompt institution of the appropriate antibiotic in adequate amounts. However, the most astute diagnosis and excellent therapeutic regimen may be frustrated by severe complicating illness. Pneumococcal meningitis is an outstanding example of these principles.

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DISCUSSION

DR. FISCHER: I would like to underscore what to me is a very interesting point, namely penicillin—tetracycline antagonism. This, I believe, was shown experimentally by Jawetz and coworkers in the test tube and in mouse infections some years ago, and I believe that your observations, Dr. Kirby, and those of Dowling and Lepper actually provide clinical support to the experimental observation and it makes me feel a little more confident about the use of mice, at least for demonstrating drug combination activity.

DR. ROMANSKY: I would like to emphasize one point, again, that Dr. Kirby mentioned in our report, and that is that probably any one of the antibiotics given in an adequate dose, individually, will be satisfactory, whether it is erythromycin or tetracycline or chloramphenicol. Also, I would point out the case he mentioned, of the patient who died of the anaphylactic shock from penicillin. There is a real problem in being unable to get a history from the patient who is allergic. This patient, who had pneumococcal meningitis, first received a large dose of aqueous procaine-penicillin, one hour later was given a million units of penicillin intravenously, and within just a few minutes developed a tremendous urticaria, starting from her feet all the way up to her head, and went into anaphylactic shock. She was brought out of the anaphylactic shock by the usual means, but two hours later went into anaphylactic shock again and expired. This is something we must be very careful of in our use of penicillin in comatose patients. We do not use penicillin in patients in coma or in those unable to give an adequate history of drug allergies.
drug allergies.

DR. ROSS: I would like to mention our experience with a case of pneumococcal meningitis in pediatrics. We use a combination of penicillin and chloromycetin. Now, it seems to me that the same objections would obtain in the use of a bactericidal and a bacteriostatic agent when chloramphenicol and penicillin are used as with tetracycline and penicillin. Yet, our results have been quite satisfactory. We have not noted any increase in our mortality rate in the pediatric age group when we combined chloramphenicol with penicillin.

MANAGEMENT OF HEMOPHILUS INFLUENZAE TYPE B MENINGITIS

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INTRODUCTION

Historically, interest in the management of Hemophilus influenzae type B meningitis began with the introduction of the sulfonamides, which offered the first hope of cure in a highly fatal disease. Survival was also improved by the use of Alexander's serum and streptomycin. The broad-spectrum antibiotics, tetracycline and chloramphenicol, however, would appear to be the most effective agents against H. influenzae type B infections, although excellent results have been reported by various authors with combined streptomycin and sulfonamide therapy [1-4]. In order to compare the results of various methods of treatment reported in the literature, they have been tabulated in Table 1. The purpose of this paper is to compare our results using three comparable treatment regimes with those in Table 1, and to present other data of interest.

METHODS AND MATERIALS

Only meningitic patients with either blood or cerebrospinal fluid cultures yielding H. influenzae type B were utilized for this study. Two hundred and thirty-five patients were treated between 1951 and 1959. Most patients were initially treated with penicillin, sulfonamides, streptomycin, and chloramphenicol or one of the tetracyclines (Table 2). Penicillin was discontinued when H. influenzae type B was identified by bacteriologic culture. Streptomycin was given intramuscularly 50 mg/kg/day for 3-5 days. Sulfadiazine was given in a dose of 200 mg/kg/day over a 7-14 day period, initially by subcutaneous clysis and then orally as tolerated. Chloramphenicol or the tetracyclines were administered in a dose of 75-100 mg/kg/day over a 10-15 day period.

RESULTS

The monthly distribution of cases can be seen in Fig. 1. The distribution of cases was fairly constant over a nine-month period; thus, cases were not restricted in large part just to the winter months.

The age distribution of patients can be seen in Fig. 2. It follows the distribution described in previous years except, perhaps, for a larger group of children over one year of age. Sex distribution revealed that 61% of the patients were male. All patients were Caucasian except for ten Negro and two Oriental children.

The duration of illness prior to admission was less than 48 hr in ninety-one patients (39%), between 2 and 7 days in ninety-four patients (40%) and over 7 days

Table 1. Results of Treatment of *Hemophilus influenzae* Meningitis by Various Groups

Reference	Therapy used	Number of patients	% Mortality	% Residual handicap
Alexander et al., 1946	streptomycin, sulfadiazine, serum	25	12	12
Crook et al., 1948	serum, sulfadiazine	52	23	13.5
	serum, sulfadiazine, streptomycin	49	17.1	18.4
Hoyne and Brown, 1948	streptomycin, sulfadiazine, serum (in 11 cases)	28	7.2	21.4
Prather and Smith, 1950	chloramphenicol	15	0	0
Applebaum and Nelson, 1950	streptomycin, sulfadiazine, serum (in 17 cases)	90	3.3	10
McCrumb et al., 1951	chloramphenicol	12	0	16.7
Ross et al., 1952	chloramphenicol, sulfadiazine	15	13	6.7
Lepper et al., 1952	chlortetracycline	18	0	5.6
	chlortetracycline, streptomycin, sulfisoxazole	14	0	14.3
Koch and Carson, 1954	streptomycin, sulfadiazine, serum	27	22	18.5
	streptomycin, sulfa, oxytetracycline	46	4.3	4.3
	streptomycin, sulfa, oxytetracycline, chloramphenicol	16	0	6.3
Moll and Stamm, 1954	oxytetracycline, sulfadiazine	23	13	0
Meade and Weinstein, 1956	streptomycin, sulfadiazine	40	0	15
Bourke and O'Dwyer, 1956	sulfathiazole, penicillin	13	61	0
	sulfathiazole, penicillin, streptomycin	19	47	0
	sulfathiazole, streptomycin, tetracycline, chloramphenicol	19	10.5	0
Koch and Hanson, 1957	tetracycline	12	0	0
Platou et al., 1959	penicillin, sulfadiazine, chloramphenicol	118	8.5	16.8 (of 113 cases)
Shaw and Bruyn, 1960	streptomycin, sulfadiazine, chloramphenicol (some)	132	6.1	6.1
	Total	783	Avg 9.9	Avg 10.3

in fifty patients (21%). One hundred and thirty-two patients (56%) received an antibiotic (usually penicillin) prior to hospital admission. The use of antibiotics before admission did not appreciably affect the ability to isolate organisms from the cerebrospinal fluid; since cultures were positive in 96% of the cases. This was not true of blood cultures except when penicillin alone was administered prior to admission. Blood cultures in 78% of the cases yielded organisms when no antibacterial medication had been administered before admission. In the group receiving tetracycline, chloramphenicol, sulfa, or streptomycin, either alone or in combination, only 40% of the blood cultures yielded bacterial growth, compared to 70% for those receiving penicillin alone.

Historical complaints were most commonly lethargy, fever, vomiting, and a spreading respiratory infection. Physical findings on admission included lethargy, stupor, and meningeal signs in 89% of the cases. Otitis media was noted in 32%

Table 2. Results in Specific Therapy Groups

Therapy groups	Total	Expired	Residual
streptomycin, sulfadiazine, oxytetracycline	50	1	4
streptomycin, oxytetracycline	8	0	0
sulfadiazine, oxytetracycline	4	1	0
oxytetracycline	1	0	0
streptomycin, sulfadiazine, tetracycline	69	8	5
streptomycin, tetracycline	2	0	0
tetracycline	9	0	1
streptomycin, sulfadiazine, chloramphenicol	27	5	3
streptomycin, chloramphenicol	2	0	0
sulfadiazine, chloramphenicol	26	3	3
chloramphenicol	3	0	0
streptomycin, sulfadiazine, oxytetracycline, chloramphenicol	9	1	0
streptomycin, sulfadiazine, tetracycline, chloramphenicol	13	0	1
miscellaneous group with all five drugs	12	0	0
Total	235	19	17

of the patients. Other infections present on admission were three cases of mastoiditis, two of periorbital cellulitis, and one case of epiglottitis.

Cerebrospinal fluid examination by stained smear revealed that 54 of 209 smears examined did not reveal organisms, but subsequent cultures in 48 of these yielded H. influenzae type B organisms. Sixteen smears were incorrectly interpreted, usually as Gram-negative diplococci or Gram-positive cocci.

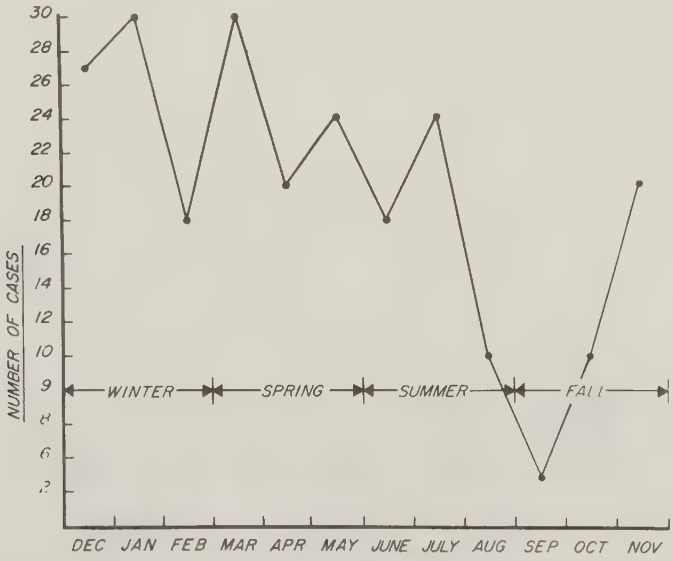

Fig. 1. Seasonal incidence of H. influenzae meningitis in 235 cases.

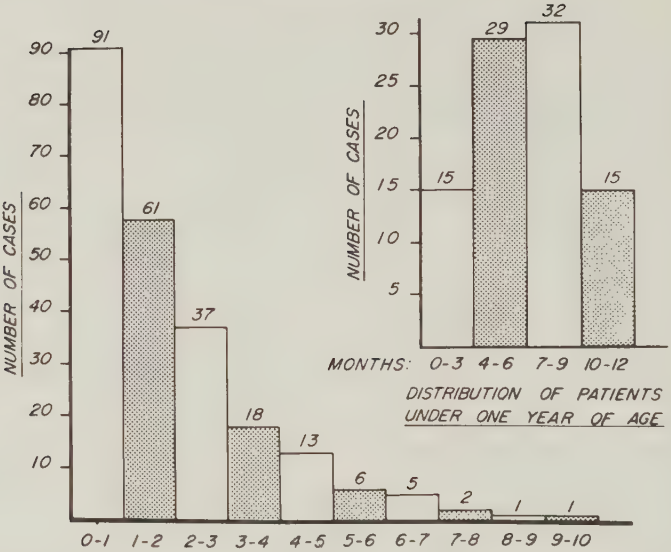

Fig. 2. Age distribution in years of 235 cases of H. influenzae meningitis.

Mortality. There were nineteen deaths (8% mortality) among the 235 patients. By tabulating the deaths according to the type of therapy, using tetracycline, oxytetracycline, or chloramphenicol along with varied combinations of streptomycin and sulfadiazine (Fig. 3), the death rates were 4.5% in the oxytetracycline group, 8.4% in the tetracycline group, and 12.5% in the chloramphenicol group. Further details concerning the exact medication used for therapy is shown in Table 2. The age distribution of the children who died is shown in Table 3. Fourteen of the nineteen deaths were over one year of age and were distributed evenly

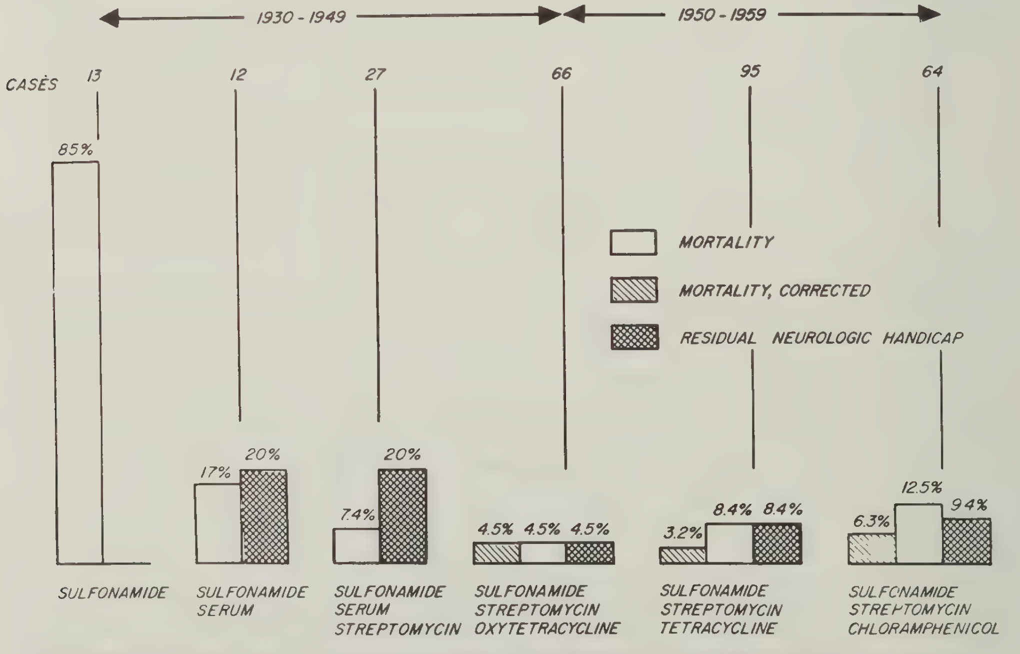

Fig. 3. Clinical results in 277 cases of H. influenzae meningitis since introduction of specific therapy.

from one to five years. Eleven of the nineteen children expired within 24 hr following admission. Three deaths occurred within 48 hr while the remaining five expired between the fourth and ninth hospital day. Autopsies were performed on seventeen of the nineteen children who expired. Five showed significant cerebral edema; three, pulmonary edema; and two, generalized edema. Four patients had marked fatty infiltration of the liver along with renal tabular degeneration. Two had massive bilateral adrenal hemorrhage, associated clinically with severe shock. Severe bronchopneumonia was found in five. Acute staphalococcal entercolitis was found in one patient treated with tetracycline. No subdural effusions were discovered, although one child had thrombosis of the saggital and lateral venous sinuses along with fatty infiltration of the liver and severe bronchopneumonia. Table 3 provides details of each death.

Seventeen patients (7.2%) were left with residual handicaps which are tabulated in Table 4. The residual morbidity rate for the different combinations of antibiotics was 4.5% for the oxytetracycline group, 8.4% for the tetracycline group, and 9.4% for the chloramphenicol group. The most severely neurologically handicapped children were usually less than one year of age and ill two weeks or more before admission (cases 9, 15, 16, and 17 in Table 2). With the exception of three children, all with residual handicaps had convulsions before admission or during their hospital stay and five had an associated subdural effusion.

Subdural effusion. Fifty-eight patients had subdural taps performed at which time thirty-two subdural effusions were discovered (16%). Twenty of the thirty-two productive taps were performed between the fourth and ninth hospital day, seven between the tenth and fourteenth hospital day, while only four taps were done between the second and fourth hospital day. One was performed on the twenty-third hospital day. There appeared to be a relationship between the number of days the child was ill prior to admission and the occurrence of subdural effusion. Only eight subdural effusions (9.2%) occurred in the 88 children ill less than 48 hr, compared to twenty-four (16.5%) in the 144 patients ill over 2 days. The indications for performing subdural taps in the children with effusions were: prolonged fever or a new elevation of temperature in seventeen, a "crackpot" sound on head percussion in three, and convulsions in three. Taps were performed on seven children described as irritable, and two taps were done routinely on children who were afebrile and presumably well. Twenty-three responded to repetitive needle aspiration, six had subdural membranes removed at surgery, and three others had a craniotomy. One of the latter showed no membranes, one revealed arachnoiditis with fluid but no membrane, and the third showed cystic degeneration of the brain without membrane formation. Only those patients not responding to needle aspiration were subjected to surgery.

Twenty-six of the thirty-two patients with subdural effusions were considered to be completely well on discharge. Of the six patients with residual handicap, development appeared to be slow in two patients, associated, in one, with a behavior problem. One child had a sixth-nerve palsy which was considerably improved several months later. Another had a right hemiplegia and a homonomous hemianopsia. In only one instance was there severe mental and motor retardation. This was in a one year old child who had received 2 weeks of treatment for otitis media before the diagnosis of meningitis was established. The sixth child had severe mental and motor retardation before his illness. Three of these six patients with residual handicap on discharge had surgery for the removal of subdural membranes.

In an attempt to ascertain their present state of health, questionnaires were mailed to the parents of the thirty-two patients who had subdural effusion. Eight-

Table 3. Nineteen Deaths Due to *Hemophilus influenzae* Meningitis

Patient	Therapy group*	Age, yr	Sex	Days ill before admission	Condition on admission	Occurrence of convulsions	Time between admission and death	Subdural effusion	Autopsy findings	Comments
1	T	2	M	21	comatose	+	8 days	–	fatty liver, cerebral edema	no response to treatment
2	T	$2\frac{1}{2}$	M	2	semicomatose	+	14 hr	–	pulmonary edema, bronchopneumonia	fixed pupils on admission
3	T	3	M	1	comatose	+	12 hr	–	cerebral edema, adrenal hemorrhage	died in shock, levophed and hydrocortisone used
4	T	$3\frac{1}{2}$	M	1	moribund	–	40 min	–	cerebral edema, pulmonary edema	fulminating course
5	T	3 mo.	M	10	semicomatose	–	3 hr	–	adrenal hemorrhage	cyanotic and in shock on admission
6	T	3	M	2	comatose	–	8 hr	–	bronchopneumonia	terminal aspiration
7	T	8 mo.	F	1	semicomatose	+	8 days	–	fatty liver, cerebral edema, cerebral purulent exudate	excessive intravenous fluids
8	T	$1\frac{1}{2}$	M	1	comatose	+	30 min	–	cerebral edema, herniation of cerebellum	fixed, dilated pupils on admission
9	O	$5\frac{1}{2}$	M	1	semicomatose	–	5 hr	–	fatty liver, cerebral edema, pulmonary edema	fixed, dilated pupils on admission
10	O	4 mo.	M	$\frac{1}{2}$	semicomatose	+	2 days	–	no autopsy	possible subdural effusion

Table 3 cont.

Patient	Therapy group*	Age, yr	Sex	Days ill before admission	Condition on admission	Occurrence of convulsions	Time between admission and death	Subdural effusion	Autopsy findings	Comments
11	O	6	F	2	semicomatose	–	7 days	–	fatty liver, generalized edema, enterocolitis	staphylococcus sepsis super-infection
12	C	4	M	10	semicomatose	–	36 hr	–	cerebral edema, generalized edema	excessive intravenous fluids - cardiac failure
13	C	5	M	2	moribund	+	3 hr	–	bronchopneumonia	fixed, dilated pupils on admission
14	C	7 mo.	M	2	semicomatose	+	24 hr	–	hemorrhage, adrenal medulla	expired suddenly
15	C	6½	M	1	comatose	+	12 hr	–	no autopsy	expired suddenly, no cause determined
16	C	1½	M	3	moribund	+	11 hr	–	bronchopneumonia, chronic meningitis	decerebrate on admission
17	C	9	M	2	semicomatose	–	9 days	–	bronchopneumonia, lateral and sagittal sinus thrombosis	apneic second day; in respirator until death
18	C	4	M	1	lethargic	–	4 days	–	cerebral edema, bronchopneumonia	cardiac arrest in 6 hr; moribund until death
19	C	5 mo.	M	8	moribund	+	13 hr	–	cerebral edema, cerebral purulent exudate	moribund on admission; excessive intravenous fluids

*T, tetracycline; O, oxytetracycline; C, chloramphenicol.

Table 4. Seventeen Patients with Residual Handicap Due to *Hemophilus influenzae* Meningitis

Patient	Therapy group*	Age, yr	Sex	Days ill before admission	Condition on admission	Occurrence of convulsions	Subdural effusion	Nature of residual handicap	Comments
1	T	2	M	2	semicomatose	–	–	ataxia, hearing loss	streptomycin 50 mg/kg for 6 days
2	T	7 mo.	F	4	comatose	+	+ with membranes	behavior disorder, slow development	
3	T	4½	M	2	comatose	+	–	strabismus, limp in outdown leg	near normal at follow-up
4	T	4	M	2	comatose opisthotonic	+	–	cortical blindness	improved at follow-up
5	T	3½	M	2	semicomatose	+	–	behavior problem	
6	T	1½	M	1	comatose	+	+	sixth-nerve palsy	
7	T	1½	F	60	comatose	+	–	slow development, right-sided weakness	
8	T	4½	M	3	comatose	–	–	limp due to pyoarthritis	improved at follow-up
9	O	6	M	30	comatose	+	–	arrested, hydrocephalus, severe retardation	

Table 4 cont.

Patient	Therapy group*	Age, yr	Sex	Days ill before admission	Condition on admission	Occurrence of convulsions	Subdural effusion	Nature of residual handicap	Comments
10	O	1½	M	1	comatose	+	+	right hemiparesis, homonymous hemianopsia	
11	O	2	M	2	comatose	+	–	behavior problem, muscle uncoordination	
12	C	4 mo.	F	5	lethargic	+	+	slow development	
13	C	6	M	2	comatose	+	–	slow development	
14	C	1½	F	1	lethargic	–	–	behavior problem	
15	C	5 mo.	M	21	comatose opisthotonic	+	–	decerebrate, severe retardation	
16	C	11 mo.	M	14	semicomatose	+	+	severe retardation	
17	C	3 mo.	M	14	comatose	+	–	severe retardation, seizures	

*T, tetracycline; O, oxytetracycline; C, chloramphenicol.

een replies were received. Follow-up was from one month to 6 years, with an average of $2\frac{1}{2}$ years. Fourteen of these eighteen patients had been considered well on discharge with no evidence of retardation. Thirteen of these fourteen patients who were considered well on discharge returned to their usual state of health and compared in development to their siblings. The one patient who was considered well at the time of discharge was normal in all respects except for a slight hearing loss in one ear, discovered on follow-up. In addition to these eighteen patients, five others were followed from one month to three years in the hospital outpatient department and were well. Thus, of twenty-three patients with subdural effusions who had follow-up, six had neurologic residual effects. This rate of at least 19% for the entire subdural group is considerably higher than the 7.2% morbidity rate for the entire group.

Complications of treatment can be divided into five groups: secondary staphylococcal infections, sensitivity reactions to sulfadiazine, leukopenia or agranulocytosis secondary to chloramphenicol, and miscellaneous.

Three patients developed staphylococcal wound infections at craniotomy sites. Two of these patients also developed an associated osteomyelitis of the bone flap. Two of these had received tetracycline and the third, chloramphenicol. One child treated with oxytetracycline expired following an acute staphylococcal enterocolitis. Another child developed an abscess of the buttock at the site of an intramuscular tetracycline injection.

Sulfadiazine reactions consisted of hematuria in five children and macular rashes in two others. These cleared completely when the dosage was decreased or the drug discontinued.

In the group receiving chloramphenicol there were nine children who developed granulocytopenia. In four of these the granulocytes were below 10% of the total white count. In only two, however, was this drop in granulocytes accompanied by a concomitant reduction in the total white blood cell count below 4000. The total white cell count and the granulocyte count returned to normal within 2 days after chloramphenicol was discontinued, and no subsequent cases of aplastic anemia are known to have developed.

Swelling of the lower leg and ankle with tenderness and increased warmth was a frequent and distressing complication of the use of venous cutdowns, especially when oxytetracycline or tetracycline was used. Other complications noted during the course of treatment were a pyoarthritis, which left some residual joint deformity, oral moniliasis, and a catarrhal jaundice, which resolved and was unexplained.

DISCUSSION

A paper from this hospital in 1955 [5] demonstrated improvement in mortality and morbidity rates since the introduction of sulfonamides, Alexander's serum and streptomycin. Further experience with the tetracyclines and chloramphenicol since 1954 is demonstrated in Fig. 3. It can be readily seen from these figures that since the introduction of the tetracyclines in 1949 there has not been an appreciable change in mortality and morbidity rates.

Shaw and Bruyn [4] as well as Meade and Weinstein [3] feel that streptomycin and sulfadiazine are the drugs of choice, although the former group utilized chloramphenicol in children with severe infection.

As can be seen in Table 1, other groups as well have had excellent results with streptomycin and sulfadiazine [1–3]. Still others [6–8], however, have not been so successful. This difference is probably due to variation in the virulence of the organism from year to year, differences in drug dosage, the varying severity and length of illness of the patients concerned, and differences in the quality of supportive care rendered to critically ill patients. Since sulfonamides alone are not very effective for treating H. influenzae meningitis [8, 5] the addition of streptomycin provides just one truly effective antibiotic. Alexander [9] has demonstrated that streptomycin resistant organisms develop within 48-72 hr by subculturing in sublethal doses of streptomycin. Instances of drug fastness to streptomycin have been found in clinical infections as well [7]. By adding either tetracycline or chloramphenicol to the combination of sulfadiazine and streptomycin the problem of resistance can be obviated.

The results presented in Table 1 suggest that mortality and morbidity rates were lower when tetracycline or chloramphenicol was used, either alone or in combination with streptomycin and sulfadiazine. At this time it is not as yet clear whether the use of tetracycline or chloramphenicol alone will be just as effective as a combination of drugs in treating this disease [10-12]. Prather and Smith [11] in 1950 reported on the treatment of fifteen children who were treated with chloramphenicol alone. All recovered and no permanent residual handicap occurred. In 1951 McCrumb and his group [10] reported no deaths among twelve patients treated with chloramphenicol alone. Deane and others [13] in 1953 treated twelve patients with chloramphenicol with one death occurring in a child whose treatment was started eight days after the onset of her illness. Lepper and his group [14] in 1952 treated eighteen patients with chlortetracycline alone without mortality. Only one child had residual handicap. In 1957 Koch and Hansen [12] treated twelve cases with tetracycline alone and had no deaths. In spite of these impressive results in small series of patients, it will be necessary to prove conclusively that single broad-spectrum therapy is efficacious by treating a larger number of cases over a period of years to minimize the possibility that these excellent results were due to a low virulence of organisms at the particular time the drug was used in the area of the study.

The fact that in our patients another antibiotic was added or substituted for the original antibiotic in thirty-four instances leads us to consider possible drug failures. These switches were invariably between the tetracyclines and chloramphenicol. The four chief reasons for major antibiotic changes were: unexplained elevations of temperature, an increase in spinal fluid pleocytosis, the substitution of chloramphenicol for tetracycline because it was considered a better antibiotic, and the substitution of tetracycline because of the development of peripheral blood changes while on chloramphenicol. There were no suspected relapses of meningitis on either a tetracycline or chloramphenicol which were proven by cerebrospinal fluid culture. New elevations in temperature were frequently later explained by the discovery of subdural effusion or thrombophlebitis. However, there were four instances where the elevation of temperature and spinal fluid pleocytosis could not be explained and where the course was suggestive of clinical relapse. There were two children in whom a relapse was suspected while on adequate dosage of tetracycline. Because of the greater interest in these cases their histories follow in detail.

A 5-month-old male infant had a 38-cc subdural effusion drained on his fourth hospital day of adequate treatment with tetracycline, streptomycin, and sulfadiazine. The effusion contained 1450 white cells/mm^3 with 74% polymorphonuclears and Gram-negative rods, which on smear gave a positive test for the Quellung reaction. Subsequent cultures, however, remained sterile. Chloramphenicol was substituted for tetracycline because of the presence of the organisms on the subdural smear and the infant made an uneventful recovery.

A 3-year-old male child treated with tetracycline had a subdural effusion drained and membranes removed through burr holes on his twenty-third hospital day. Two days postoperatively he had a temperature elevation and lumbar puncture revealed 100 WBC, 83% polys. No organisms were seen on smear and none were grown on culture. Chloramphenicol and sulfadiazine were substituted for tetracycline and the child did well.

Also, there were two children on adequate dosage of chloramphenicol in whom a relapse was suspected. Their clinical courses follow.

A 5-month-old female was treated with streptomycin, sulfadiazine, and chloramphenicol. Subdural effusion was successfully drained and the infant was discharged in good condition after an apparently adequate course of treatment for 2 weeks. Her discharge lumbar puncture showed 166 WBC. Four days after discharge she developed fever and repeat lumbar puncture revealed 327 WBC. The smear was unrevealing and the culture was sterile. Subdural taps were dry. Because of the increased cerebrospinal fluid pleocytosis tetracycline therapy was initiated with complete recovery.

A $4\frac{1}{2}$-year-old male was treated for 8 days with tetracycline. Because of an unexplained fever, chloramphenicol was substituted and given for 9 days. On discharge cerebrospinal fluid revealed only 15 WBC. Seven days later he was readmitted with fever and a stiff neck. Spinal fluid contained 460 WBC, 58% polys. Smear and culture were negative. He was retreated with chloramphenicol for 14 days and made an uneventful recovery.

In reviewing cases in the literature it is unusual to find bona fide cases of relapse after treatment with the tetracyclines or chloramphenicol. McCrumb [10] mentions a child who was treated with effective doses of aureomycin but in whom the Hemophilus organism reappeared in the spinal fluid. The child was then treated with chloramphenicol and the spinal fluid became sterile only to become reinfected. Chloramphenicol again eradicated the infection. Deane [13] reported the case of a 9-month-old infant treated with chloramphenicol who had the organism present in his spinal fluid throughout his hospitalization until death.

Our patients presented three nearly distinct clinical patterns in the development and course of their meningitis. The first pattern concerns children who recover completely with no discernible residual handicap. It is unfortunate that it was not possible to do a close follow-up on this group for it is quite probable that children who were considered well on discharge may well develop signs of neurological handicap in later life. Bergstrand [15] and his group in 1957 did a follow-up of their 37 cases of H. influenzae meningitis treated from 1947 to 1954 and found an additional nine children with residual handicap. This is a small series of cases but this residual rate of 24% is considerably higher than our residual rate of 7%. The fact that children ill between 2 and 7 days before admission had the lowest mortality rate (1%) and the lowest residual rate (3%) stresses the importance of low organism virulence and high host resistance.

The second pattern concerns children with fulminating disease who expire shortly following admission in spite of antibiotic therapy. Convulsions are common in this group and death occurs before subdural effusion develops (Table 3). The most important factors operating in these cases appear to be high organism virulence, overwhelming infection, and poor host resistance. Ten of the nineteen patients who expired were apparently beyond any help from therapeutic measures. Four children were moribund on admission and expired within 13 hr (Cases 4, 13, 16, 19). Two children had massive, bilateral, adrenal cortical hemorrhage associated with shock and they expired within 12 hr of admission, although adrenal steroids were used (case 3, 5).

One child who expired suddenly within 24 hr of admission had adrenal medullary hemorrhage at autopsy (case 14). Another child expired 30 min after admission with fixed dilated pupils. At autopsy he had herniation of his cerebellum through the foramen magnum and severe cerebral edema, as well. The herniation appears to have antedated the diagnostic lumbar puncture since he was moribund on admission. The remaining two children had fixed dilated pupils on admission and expired with no improvement within 14 hr. In these ten children it does not appear that a different antibiotic regime or more effective supportive care could have altered the final outcome.

The post-morten findings in cases 1, 7, 11, 12, 17, and 19 (Table 3) suggested the possibility that iatrogenic factors may have played a part in their death. Cerebral edema may have been aggravated by the infusion of excessive intravenous fluids in cases 7, 17, and 19. The marked degree of fatty infiltration of the liver found in the autopsies of cases 1, 7, and 11 suggested that there may have been a relationship to tetracycline therapy. The problem of superinfection brought out by case 11 has been discussed. In case 12 there was no doubt that excessive intravenous fluids contributed to his death. Cardiac failure, gross tissue edema, and marked cerebral edema were found at autopsy.

Fatty infiltration of the liver following the use of tetracyclines has been reported [16–19]. In three of the four patients in this series who developed this complication intravenous oxytetracycline or tetracycline was administered in a dosage of 100–150 mg/kg/day over a 7–8 day period. This is a larger dosage than recommended and was inadvertently administered in these three patients. Cases 1, 7, and 11 showed no clinical evidence of liver damage but the degree of fatty changes found at autopsy suggested possible deleterious effects on the liver. Experimentally, however, permanent liver damage does not occur as instances of fatty change are completely reversible when tetracycline is discontinued [20–22]. In reviewing autopsy findings in deaths at the Childrens Hospital due to H. influenzae meningitis before the use of the tetracycline drugs, instances of significant fatty infiltration of the liver were found so it is not entirely a drug-induced problem.

The third pattern concerns the child who recovers but is left with residual handicap. This child's length of illness before admission lies at two extremes; he either has been ill only 2 or 3 days before admission, in which case his residual handicap may not be severe, or he has been ill 2 weeks or more in which case he is usually severely retarded (Table 4). Coma, subdural effusion, and convulsions are more common in this group of patients.

The residual handicap rate of 7.2% compares favorably with reports in the literature (Table 1). In 1953, from Childrens Hospital of Los Angeles, Smith [23] reported a residual handicap rate of 7.6% with 106 patients receiving varied combinations of oxytetracycline, streptomycin, sulfadiazine, and Alexander's serum. In 1960, Shaw and Bruyn [4], from San Francisco, reported a residual handicap rate of 6% when 103 children were treated with streptomycin and sulfonamides, and with chloramphenicol in some instances. Also, in 1959, Platou [24] and his group reported a residual morbidity rate of 16% in 113 patients.

In reviewing our cases of residual handicap, three clinical features were evident which may have been important contributing factors to the development of residual handicap. These factors are the length of time the child was ill before admission, the severity of the illness, and the presence of subdural effusion.

There is no question that children who were ill for prolonged periods of time before admission as in cases 7, 9, 15, 16, and 17 (Table 4) were the most severely retarded.

The incidence of subdural effusion in the group with residual handicap is 30% (5 of 17 cases), suggesting that the pathological process inducing formation of subdural effusions may be the same as that causing neurological handicap. McKay and his group [25] reviewed 75 cases who were admitted between 1945 and 1948, before subdural taps were a routine procedure. They noted that of eighteen children in this group with gross neurologic and mental deficits, sixteen would have had subdural taps performed using the criteria they now utilize. In the follow-up study of Bergstrand [15] which was previously mentioned, no subdural taps had been performed in a group of 77 patients with a residual rate of 20% who had been followed for over one year after the onset of their acute purulent meningitis. Platou and his group [24] found that serious disability was frequent among those children who had early subdural effusions.

Another factor which appears to be related to residual handicap is the degree of toxicity of the child's illness on admission. The majority of these children with neurological sequellae were comatose on admission. As convulsions were frequent in this group as well, their condition on admission occasionally represented a postconvulsive state. It is most probable that the convulsions are a result of the same pathological process that produced the residual handicaps.

We cannot add any new facts as to the cause of subdural effusions; however, there appeared to be more in the group of children ill over 2 days before admission (16.5%) than in those children ill less than 2 days before admission (9.2%).

The indications for performing a subdural tap have not changed since McKay [25] and his group first focused attention on this complication of purulent meningitis. In 1951 Smith et al. [26] found twenty subdural effusions in 43 patients less than two years of age who were tapped. In nine of these patients the subdural tap had been done routinely without clinical indication for the tap. Many clinicians are reluctant to do a subdural tap on a child who is doing well clinically, and it has been our policy to tap only when indicated. It may be that further study will show that all children with purulent meningitis and accessible fontanels should have subdural taps done regardless of their clinical course. Since most of our children with subdural effusions were between the ages of 4 and 9 months (only one subdural effusion was found in a child over two years of age), this means that either children with open fontanels are more prone to develop effusions or they are missed in children with closed fontanels. As pointed out by many authors, we simply do not know the incidence of subdural effusions in children with purulent meningitis with closed fontanels, but one wonders at their clinical significance if they are so small as to remain undiscovered.

In a follow-up of twenty-three of our thirty-two patients with subdural effusions, only one additional instance of residual handicap was discovered—a child with a slight hearing loss which had not been noted on discharge. The remaining 22 children who did not have residual handicaps on discharge from the hospital compared favorably with siblings and appeared in good health. This suggests that adequate treatment of subdural effusion offers assurance that the child who is discharged in good condition will not demonstrate later residual effects.

SUMMARY

Two hundred and thirty-five children with Hemophilus influenzae type B meningitis were treated over a 10-year period with varied combinations of strep-

tomycin and sulfadiazine plus either oxytetracycline, tetracycline, or chloramphenicol. The mortality rate for the over-all group was 8%, the residual handicap rate was 7.2%, and the subdural effusion rate was 13.6%. There did not appear to be a significant difference in the mortality or residual handicap rates among the children in the three antibiotic groups, although the lowest mortality and morbidity was in the oxytetracycline group. Death due to H. influenzae meningitis could rarely be attributed to drug failure. Eleven of the nineteen deaths were moribund on admission and appeared to be beyond therapeutic help. Careful attention must be paid to proper supportive measures. The overzealous use of intravenous fluids must be guarded against and the importance of adequate nursing care is stressed. A suggested regime of treatment is offered.

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DISCUSSION

Q: What are your criteria for cessation of therapy?

DR. BOVE: Usually about 7 days of afebrile state.

Q: Is that the only one you have beside spinal fluid cultures?

DR. ROSS: I would like to make just two points from our own experience with H. influenzae meningitis in children. First of all, we favor chloramphenicol as our prime drug in the treatment of this disease for two reasons. About 30 to 40% of the coexisting chloramphenicol blood level appears in the spinal fluids. Chloramphenicol is a small molecule and it, unlike tetracycline, traverses the blood-brain barrier very well. Secondly, there have been few if any chloramphenicol resistant strains of H. influenzae encountered thus far. I would agree with Dr. Bove that combination therapy is very much in order, however, I would be in favor of omitting streptomycin since one can achieve extremely good results employing chloromycetin and sulfonamides, and indeed the work of McCrumb and Woodward indicates that chloromycetin alone will yield equally good results.

COMPARATIVE ACTION OF SPIRAMYCIN AND DARAPRIM IN THE TREATMENT OF HUMAN OCULAR TOXOPLASMOSIS*

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INTRODUCTION

The cases selected for this study presented with active retino-choroidal lesions considered toxoplasmic in origin, an important etiology of posterior granulomatous uveitis. The agent of ocular toxoplasmosis is a protozoan, Toxoplasma gondii, an obligatory intracellular parasite. The parasite usually lodges within the retinal cells of the eye causing a coagulation necrosis. Some of these lesions certainly resolve spontaneously and may be discovered many years later on routine examinations as old chorioretinal scars. However, it is safe to say that the clinically active lesions seen by the ophthalmologist would generally result in blindness or greatly dimished visual acuity in the absence of early and effective therapy. This inflammatory process of the eye involving the retina and the underlying nutritive vascular layer, called the choroid, occurs mainly in young individuals under forty years of age. The acute disease may evolve within a few months to permanent loss of vision in the affected eye, whereas with successful therapy resolution of the infection is usually complete in about eight weeks. It is difficult to determine, with any degree of certainty, the etiological diagnosis of a retino-choroidal lesion. Reliance must be placed on indirect evidence as direct examination of the intra-ocular fluids demonstrating protozoa or other organisms require specialized techniques and personnel which are usually not available. Rapid response to specific therapy is a strong argument in favor of a particular etiology. In the literature highly variable estimates of the importance of toxoplasmosis as an etiological factor in retino-choroidal lesions have been made. These range from 20% to 70% [1]. Our cases with acute retino-choroidal lesions were chosen on the basis of a positive Sabin-Feldman methylene-blue dye test for toxoplasmosis in dilution of 1:16 or more, elimination of other probable etiological factors by complete diagnostic survey, and a history of exposure to reservoirs of toxoplasmosis in nature, at home, or at work.

Pyrimethamine (Daraprim) and some of the sulfonamides (sulfamerazine, sulfadiazine, sulfamethazine) were found to be exceptionally active against experimentally induced toxoplasmosis in animals and association of the two drugs resulted in a synergistic action [2-7]. Bogacz in 1954 [8] and Garin [9] in 1956 reported in Copenhagen on the effectiveness of spiramycin (Rovamycine, Poulenc) against acute induced toxoplasmosis in rabbits and mice. At that time Garin supplied Eyles with samples of spiramycin and they later published their experimental results together, confirming the above data [9].

METHODS AND RESULTS

In the Uveitis Clinic of the Wills Eye Hospital in Philadelphia, from June 1959 to the present, twenty cases of acute posterior toxoplasmic uveitis were given

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*This study was supported by a grant from the Hartford Foundation. We would like to thank Dr. Guy Marier of Poulenc Limited, Montreal, Canada, for generously supplying the spiramycin (Rovamycine, Poulenc) used in this investigation.

Daraprim (loading dose 100 mg, maintenance dose 50 mg daily) and a combination of sulfonamides, whereas seventy-seven patients* were treated with spiramycin. The retino-choroidal lesions of seventeen Daraprim patients (85%) and sixty-nine (89%) spiramycin patients were rendered inactive. Eight (40%) of the Daraprim group improved in visual acuity, whereas seven (35%) retained good initial vision. Among the patients given spiramycin, fifty-six (72.7%) sensibly improved in visual acuity. The cases with no visual gain were complicated by macular lesions, opaque vitreous or retinal detachment present at the time the patient was first admitted. The majority of cases treated with Daraprim and sulfonamides presented with active satellite foci of old cicatricial chorioretinal lesions, where clinically one might suspect a high degree of immunity and a low grade virulence of the toxoplasma. Allergy to toxins may play a major role explaining the effectiveness of associated steroid therapy in some of these cases. All the patients receiving spiramycin had acutely active retino-choroidal lesions. Twenty-six had been treated unsuccessfully with steroids and antibiotics, but improved markedly when given spiramycin. In fifty-five cases on spiramycin, methylprednisolone (8-12 mg daily) was associated with the antibiotic at the onset of treatment. The dose of steroid was rapidly decreased to approximately 6 mg daily within about two weeks. The posterior uveitis could be considered inactive, in the great majority of cases, at the end of the average period of therapy, which consisted of administering 2 g of spiramycin in single daily doses for six weeks.

COMPARISON OF RESULTS

The evaluation of different therapies in clinical medicine is rendered difficult for the following reasons: lack of adequate controls; lack of comparability in some cases as to immunity, virulence of organism, etc.; variations in associated medication; and choice of cases when etiology is difficult establish. The cases treated in the above series with Daraprim and sulfonamides or spiramycin were almost certainly cases of toxoplasmic uveitis and did well under both forms of therapy. The steroids were helpful in controlling excessive inflammatory or allergic reactions in the eye which might result in irreparable damage, but could not in themselves bring about permanent cure. The twenty-six cases mentioned previously, which were treated on steroids alone without amelioration, as well as a long-term study of a series of fourteen cases of posterior uveitis treated with ACTH and fever therapy [10], attest to the absence of cures without specific therapy. Examples of the failure of steroids to ameliorate toxoplasmic uveitis in any permanent or regular fashion are frequently seen. The experienced ophthalmologist can tell with ease which case may undergo spontaneous cure. We must therefore conclude that the cases of uveitis treated with pyrimethamine and sulfonamides or spiramycin were rendered inactive as a result of the specific therapy and there can be no doubt that in these cases spontaneous cures would not have occurred. Four relapses were noted among the two series of patients studied up to fourteen months.

The advantages in using spiramycin instead of Daraprim and sulfonamides are as follows: perfect tolerance, ease of administration and cooperation of patient, predictable therapeutic effect, and no contraindication to association with other medication or physical status of patient. In three acute cases of posterior uveitis treated with pyrimethamine and sulfonamides the evolution of the inflammatory process could not be controlled satisfactorily in spite of associated steroid therapy. The lesions responded well and rapidly became inactive when spiramycin was substituted for pyrimethamine and sulfonamides. With pyrimethamine, numerous side effects, such as thrombocytopenia, granulocytopenia, dermatitis, nausea, vomiting, headache, and gastric fullness are observed (Table 1). Cooperation on the part of the patient may be difficult to obtain. In addition the blood levels of pyrimethamine are often variable, depending upon the loading dose of approximately 150 mg on the first day of administration [11]. In the presence of vomiting and nausea, adequate blood levels are not always attained. With spiramycin no loading dose is required and a single daily dose of 2 g for six weeks is sufficient in most cases. Therapy never has to be discontinued because of side effects. Spiramycin is unique among antibiotics in its greater tendency to become fixed in tissues. The toxoplasma multiply within the cells of the retina. We may theorize that the effectiveness of spiramycin in ocular toxoplasmosis may be partly explained by fixation of the antibiotic in the retina, which is preferable to a high aqueous or vitreous level. The successful and extensive clinical trial in human ocular toxoplasmosis is in opposition to the statement made by Furgiuele et al. [2] that systemic administration of spiramycin was of no value in intraocular infection since the drug penetrated only in small amounts into the aqueous and vitreous humor. Tissue affinity will have to be considered more important than intraocular penetration in the evaluation of present and future drugs for the treatment of human ocular toxoplasmosis.

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*Includes 67 patients reported by the authors in "Treatment of Human Ocular Toxoplasmosis with Spiramycin". A.M.A. Arch. Ophth. 65: 401-409, 1961.

Table 1. Complications During Treatment of Ocular Toxoplasmosis

Complications	With Daraprim, 20 patients		With spiramycin, 77 patients
	Number	%	
Nausea	20	100	0
Vomiting	10	50*	0
Headaches	16	80	0
Thrombocytopenia	2	10	0
Granulocytopenia	4	20	0
Dermatitis	1	5	0

*All females.

SUMMARY AND CONCLUSIONS

Spiramycin, and pyrimethamine combined with sulfonamides are both effective in the treatment of human ocular toxoplasmosis. However, spiramycin is to be preferred because of its perfect tolerance and predictable therapeutic effect.

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DISCUSSION

Q: Did you ever see acute retro-bulbar neuritis due to the toxoplasma?

DR. CHODOS: The cases in which neuritis occurs in toxoplasmosis are usually associated with the acute retino choroidal lesions, and are presumed to be a toxic effect on the optic nerve. It is not infrequent to see edema of the optic nerve associated with those white fluffy lesions which are in the acute active stage. It is for this reason as well that steroids are employed in order to prevent any permanent damage to the optic nerve.

Q: Have you seen it without associated retinal involvement?

DR. CHODOS: No, I have never seen a case.

Q: Since the malaria parasite has become readily resistant to Daraprim, I wondered whether this also occurs with toxoplasmosis.

DR. CHODOS: Clinically, the results with Daraprim are sometimes difficult to predict. Whether or not this is due solely to blood levels or to resistance of the organisms is difficult to ascertain. There are papers in the literature attesting to the variability and virulence of the different toxoplasma strains but it has not been our clinical experience to note any such phenomena as you have mentioned.

Q: I wonder if you have any information on the effectiveness of erythromycin, oleandomycin, and other antibiotics?

DR. CHODOS: Yes, many of the antibiotics have been tried against toxoplasmosis. The most effective antibiotic known up to the present time is spiramycin. All the others are much less effective. The tetracyclines have been found to be active as well, but none approach the activity of spiramycin among the antibiotics. The explanation for this is difficult and might be found in tissue fixation. This is the only explanation I have to offer.

PROBLEMS CONCERNED WITH CLINICAL LABORATORY TESTING AND THE USE OF ANTIBIOTICS

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INTRODUCTORY REMARKS

From the time of their introduction we have heard much about the "use" and "abuse" of antibiotics. There are almost as many varied opinions as there are persons who have ventured to make them. A conference such as the present one can thus be very useful; it allows for the sharing of the experiences of many. This can be extremely important, for no one lives long enough to make all of the mistakes himself.

This panel, as suggested by its title, will be limiting its discussions to problems concerned with testing in the clinical laboratory and the use of antibiotics. To understand these problems fully one must appreciate that the action of an antibiotic is a direct one against the invading organism itself; it does not stimulate any of the body defenses or mechanisms which combat invading organisms. There have been a number of theories concerning the mode of action of antibiotics; some of these were discussed earlier this afternoon. These will not be discussed here, but the important points to remember are that the action of an antibiotic is a direct one and to be effective for any given treatment the causative organism must be susceptible to it, and that the antibiotic must reach the site of infection in active form and sufficient quantity. There are, therefore, two points involved. The first is the sensitivity of the organism and the second, but equally important, is the pharmacodynamics of the antibiotic. Will it reach the site of infection? Does it have affinity for the tissue where the infection lies or is it a drug which will be eliminated from the body too rapidly to be effective in clearing up that particular infection? We will be touching on both of these problems today, but we will be paying particular attention to susceptibility testing by the laboratory.

The ever-increasing number of antimicrobial agents has added materially to the headaches involved in the determination of the susceptibility of an organism. Today, in Canada and the United States, we have some 28 antibiotics as follows:

> bacitracin, carbomycin, chloramphenicol, colistin, cycloserine, erythromycin, framycetin, gramicidin, kanamycin, neomycin, novobiocin, oleandomycin, paromomycin, penicillin [penicillin G, penicillin O (sodium and potassium), penicillin V, penicillin 152, dimethoxyphenylpenicillin, etc.], polymyxin, ristocetin, spiramycin, streptomycin (dihydrostreptomycin), tetracycline (chlortetracycline, oxytetracycline, demethylchlortetracycline, pyrrolidinomethyltetracycline, tetracycline as the HCl or phosphate complex), tyrothricin, vancomycin, viomycin, the antifungal antibiotics amphotericin B, cycloheximide, griseofulvin, nystatin, pimaricin, and an antitrypanosome, puromycin.

In addition we have the nitrofurans and a seemingly endless number of sulfonamides. Because of the large number of antimicrobial agents and other practical considerations, the disc method has become the preferred method for susceptibility testing of routine public health and hospital laboratory specimens. In many laboratories it is the only method used. Whether or not it is the best method is beside the point; the fact is that it is the method most of us use and are dependent on. The results of our tests will therefore depend largely upon the reliability of the discs—their identity, potency, and performance. For this reason the panel will be putting some stress on this aspect.

Impregnated discs available commercially have not always given satisfactory performance and have at times been responsible for erroneous results. To overcome this, special regulations have been put into force in Canada and, similarly, regulations have been proposed for the United States. In Canada, prior to distribution of discs, a manufacturer is required to show that his methods of manufacture and control are capable of producing uniform and satisfactory discs. Prior to release each lot is tested for potency by an extraction technique and for performance. The individual discs must not vary by more than ±40% of the labelled potency and for satisfactory performance must release antibiotic within 5 min after being placed on the agar plate. Regulations for the control of discs are now being considered in the United States. Mr. Kirshbaum, our second speaker on the panel, will be telling you about these.

The first speaker, Dr. Gerwe, Head of the Biological Control Departments, Eli Lilly and Company, will speak on "Problems Encountered in the Manufacture of Commercial Sensitivity-Test Discs," and the next speaker, Mr. Kirshbaum, Director of Control Laboratories, Division of Antibiotics, Department of Health, Education, and Welfare, will speak on "The Governmental Control of Antibiotic Discs." Dr. Anderson, the third speaker, who is Associate Professor in the Department of Microbiology, Temple University School of Medicine, will give an account of his "Evaluation of Antibiotic Susceptibility-Testing Methods," and Dr. Lind, Director of the Sias Laboratories of Brooks Hospital, will attempt to correlate "susceptibility tests" with "clinical response."

PROBLEMS ENCOUNTERED IN THE MANUFACTURE OF COMMERCIAL SENSITIVITY DISCS

E. G. Gerwe

Eli Lilly and Company, Indianapolis, Indiana

Today, I will be talking about the so-called laboratory aids or laboratory devices. I hope also to have some liberal comments from the floor. In such discussions, one should keep the definition of terms in mind because we can run into legal problems, particularly with the Food and Drug Administration. There is a question right now as to whether these legal aids are properly covered by the Food and Drug Act. That is something that will have to be determined. For in one sense they are not medical devices, they are really laboratory testing devices.

Now, as those of you in the laboratory who make use of them know, there are a number of such preparations. The simplest ones, of course, are the single discs. I think we are extremely fortunate today because as I look through the audience I see that we have representatives from all except one company that makes these laboratory aids and, of course, we have our good friends Dr. Kirshbaum from the Food and Drug Administration and Dr. Greenberg from the Canadian Department of Health plus a good sprinkling of clinicians and investigators, so we can really attack this problem from all angles. To bring you up to date, the single disc is made available by the Difco Laboratories in Detroit, the Baltimore Biological Laboratories in Baltimore, the Bio-Test Company in Omaha, Nebraska, and a number of other laboratories. In addition, the Reed and Carnrick Company have made tablet discs but I don't know whether they still make them. I did learn at this meeting from one of our good friends from Canada, Mr. Thompson, that the British Drug House prepares a very nice looking tablet which is said to have some advantages. Then, of course, there are the multiple discs, which are very well known, put out by the Consolidated Laboratories under the trade name Multi-disc and by Difco as Uni-discs. Then there is still another device, a plastic plate, the lid of which has inverted pegs. On these pegs there are measured quantities of antibiotics, so that in actual use the pegs come in contact with the agar and perform the same sort of function that the discs do.

I think we should examine closely the purpose of these discs. Unfortunately, we Americans tend to be lazy and we look for gadgets for nearly everything we do. These discs are gadgets. Everyone recognizes that this is not the best way to determine the susceptibility of an unknown bacterium or of a mixture of bacteria to a group of antibiotics. I think most of us would agree that the tube-dilution method is more accurate than the disc method, even though a good correlation has been established between the two techniques. Tube-dilution tests are time-consuming and necessitate much more elaborate preparation than is required for disc tests. Discs were never intended to give quantitative results and every manufacturer of discs stresses this point in their literature, pointing out that they should not be used quantitatively.

There are a great many factors which influence the results obtained with the single disc, the multiple disc, or the tablet and I'll outline a few of these. Some of them have been touched upon this morning. All, of course, relate to and have influence on the degree of precision of the test and on the degree of its reproducibility.

First, one is concerned with the physical characteristics of the paper used in the preparation of the discs. Incidentally, most of my discussion will relate to paper discs rather than tablets because I know very little about the preparation of tablets or about the preparation of the plastic arrangement mentioned before. Second, there are ways that one can make these discs quite precise with respect to concentration. The point touched upon by Mr. Fowler this morning related to the stability of these products. These are supposed to be scientific preparations. They should be stored under proper conditions. They cannot be abused and be expected to perform properly. Then, too, there are a great many other factors which may exert a profound effect on the results, for example, the culture medium used, the size and density of the inoculum, the incubation conditions, and, perhaps a bit on the minor side but nevertheless sometimes important, the amount of moisture in the medium.

I would like to talk a little bit about production problems. When discussing production problems we are, of course, directly concerned with their control. While the report this morning pointed out some of the failings of our present methods, there has been a marked improvement in the quality of these laboratory devices during the past several years. They have not been neglected. We do very properly get impatient sometimes and would like to see some of the problems solved much more quickly than they are. The manufacturers, on their part, have been doing a great deal of work in attempts to improve the discs. The Food and Drug Administration has also taken an active part in this work. They have, however, been limited in the past by the fact that their control authority covered only the seven certifiable antibiotics. The other 21 antibiotics mentioned by Dr. Greenberg are not covered. However, they have extended their control to include noncertifiable antibiotics that are packaged with certifiable antibiotics. These are matters that require further consideration.

About two years ago at a meeting held in Washington with the Food and Drug Administration, and which included manufacturers, representatives, and other interested people, it occurred to us that we did need some kind of an over-all control such as would be afforded by inclusion in the U.S.P. We asked Dr. Lloyd Miller whether or not sensitivity discs could be included properly in the U.S.P. At the U.S.P. Revision Committee meeting in New York last week, we were told that the U.S.P. Committee on Scope is now considering the addition of sensitivity discs.

As mentioned earlier, the problems concerned with the production of discs include satisfactory control procedures. These are of concern not only to the disc manufacturer and the distributor but also to the government people who are responsible for the over-all control of antibiotics. The distributor can be vitally concerned, for practically every manufacturer of antibiotics, such as my company, buys discs for distribution (usually free of charge) to prospective users of their individual antibiotic. The distribution of faulty discs under these circumstances could be a serious handicap to the promotion of their product.

Finally, we come to the very critical problems which confront the clinician when he uses or attempts to interpret the results obtained by the disc test. He has every right to demand that the results obtained be reliable. We are dealing with human lives and we should take any step necessary to better the precision of this test, as simple as it is. I'd like to impress upon each and every one of you one point which in my opinion is the crux of this whole problem. Many of the difficulties which have arisen in the conduct of the test have been a direct result of its being such a simple test. In spite of the many problems concerned in the manufacture of discs they are relatively simple to use. That's one reason, of course, why they have such a strong appeal to the clinical bacteriologist. This simplicity may lead to carelessness in the storage and use of discs. We must keep in mind that discs must be properly kept and also, and this is extremely important, that the results must be interpreted properly. Certain specialists, some of whose names were mentioned this morning, i.e., Dr. Kirby, Dr. Bondi, Dr. Branch, and others are able to get reproducible and reliable results. But, when their carefully studied and practiced methods are turned over rather haphazardly to less informed and less capable laboratory workers, the results may be subject to considerable error. The physician, by insisting that results be reported in zone size, can add substantially to such errors.

I want to touch briefly upon the manufacturer's problems. Please keep in mind that this covers the manufacturer of several different kinds of these disc devices. I don't personally have the details of the manufacturing steps and I hope that if questions on this aspect do arise this afternoon some of the producers will come to our rescue.

Filter paper is used in the manufacture of paper discs and it is quite obvious that such paper is not a scientifically prepared material. Very early in our investigation, when we tried to get reproducible results, it was determined that even when a great many discs from the same lot and of the same concentration of antibiotic were used on the same plate, variations occurred and that these variations could be very easily attributed to variations in the paper itself.

For example, when one took unimpregnated paper discs roughly 7 mm in diameter and weighed them individually one would find weight variations ranging from 3 to 4% up to as high as 11%. This may or may not be significant but one could rightly assume that a paper disc weighing 11% more than another would absorb correspondingly more moisture and therefore have a heavier concentration of antibiotic.

In our second experiment we took several hundred discs and dipped them in liquid one at a time following a standard procedure. Free moisture was removed by touching the discs to dry glass and they were then weighed individually. Variations in absorbency of up to 18% were found. The exact effect the difference in volume might have on the result produced by such a disc is unknown. There are a great many things about the physics of the operation of discs that we don't understand. I have in mind, for example, that in the cup, plate, or cylinder method the volume of antibiotic placed in the cup apparently makes very little difference. The important thing under the conditions of that test is the concentration of antibiotic used.

We have some excellent laboratories in this country that do routine microbiological assays of antibiotics and of vitamin preparations by biological techniques. None of these laboratories would dare attach the significance to their results that is given to the single-disc test by clinical laboratory workers. To increase the accuracy of our test we would have to expand it considerably. Also, we would have to have control discs, which are not available to us, in order to establish a response curve.

Those of us in the laboratory interested in this problem have found that discs can be made with varying degrees of precision. For example, if one takes individual discs and impregnates them, using an accurately calibrated pipette with a carefully controlled solution of antibiotic, a much more accurate disc would result than if one merely dipped the discs into a solution or if some other method, such as is used commercially, were used. In this country at least, because of high labor costs, the inoculation of individual discs is impractical.

In closing, I just want to point out again that these problems are being actively studied not only by the manufacturer but by the producers of antibiotics as well. They have a great stake in this problem. The Food and Drug Administration has done a very good job but they have had legal limitations. There has been a question, as some of you may know, as to whether or not the control of such devices is the legal responsibility of the Food and Drug Administration. At one time sensitivity discs were exempt from regulations. We have at the moment proposed regulations, apparently in their final stages, which will require the certification of discs made from certifiable antibiotics and of discs that are not certifiable but which are packaged in combination with certifiable antibiotics. We have a very active committee in the Biological Section of the Pharmaceutical Manufacturers Association studying this problem. Dr. Vera, of B.B.L., is chairman of this group and has been working together with representatives from Difco. Both laboratories are members of the P.M.A. group. The last word I had from her was that some 20 or 25 individuals were working with her on this problem. I would therefore like to leave this thought with you. We appreciate the fact that this is a very serious problem and that the manufacturers and the government people are trying their best to improve these products.

I will look forward eagerly to Dr. Wright's and Dr. Branch's report tonight on the recent WHO meeting in Geneva concerning antibiotic-sensitivity test discs. Thank you.

GOVERNMENT CONTROL OF ANTIBIOTIC SENSITIVITY DISCS

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In order that the clinical laboratory may attach significance to the results it obtains with discs, it is imperative that the disc be standardized, reliable, and of good quality. The disc is the foundation upon which the clinician selects the best drug to treat an infection and if it is unreliable the results may not be satisfactory.

Over ten years ago antibiotic discs first made their appearance. At that time the Food and Drug Administration did not believe that certification was necessary to insure their safety and efficacy of use. Therefore they were exempted from certification. However, their unprecedented use by clinical laboratories required our investigation of their quality on a large scale in 1957. Not only was this investigation a result of our own growing interest and awareness of their widescale use, but numerous members of the medical profession had expressed to us their concern over the quality of available commercial discs. As a result of this investigation it was felt that more rigid control was necessary.

First of all we must consider two separate aspects of government control, administrative and technical. The first category requires the government to act as a referee because there must be some standardization of nomenclature, labelling, and concentrations. For example, in 1957 penicillin discs were available in the following low concentrations: 1, 1.5, 2, and 5 units. Each concentration was claimed by the manufacturer to be that which would demonstrate a sensitive organism. The position of the Food and Drug Administration here is not dictatorial. The manufacturers were not told arbitrarily which concentration they had to manufacture. At a meeting of the Food and Drug Administration with representatives of disc manufacturers, basic antibiotic manufacturers, and interested members of the medical profession, the majority of those present agreed that minimum and maximum concentrations for discs should be established for the majority of the antibiotics. The two concentrations chosen for each antibiotic were those indicated by the weight of available clinical data. If additional experience should prove that any of these concentrations should be changed, this, of course, would be done.

Another aspect of administrative control is the expiration date. The Food, Drug, and Cosmetic Act requires that the expiration date be marked on the container. Furthermore, the date must be based upon actual stability data showing the discs to be stable for the length of time indicated.

Manufacturers must also submit their labels and brochures to the Food and Drug Administration for review. We check them for compliance with the law as well as for any technical errors. While the Food and Drug Administration does not specify any one test for the use of discs in the clinical laboratory, we do review the manufacturers' instructions for use to ensure that they are not misleading.

Another example of administrative control is the nonrecognition of combination discs. This is based upon the fact that there is no evidence to indicate that anything can be demonstrated with a combination disc that cannot be demonstrated with discs containing single antibiotics. In fact the use of combination discs could be misleading and confusing, to say the least.

We now come to the consideration of technical control. This, of course, is actual laboratory testing by the Food and Drug Administration. The regulation requires manufacturers to send a sample of each batch of all certifiable antibiotic discs to us for testing. If noncertifiable antimicrobial discs are to be sold in the same package, they too must be included. Each batch must pass potency and uniformity tests in order to be certified. Later, as more experience is obtained, moisture limits will be established and samples will be tested to see that they do not exceed these limits. In addition, both certifiable and noncertifiable antibiotic discs are picked up at random by Food and Drug Administration inspectors for testing.

Samples are not tested as they are in the clinical laboratory. As mentioned earlier, in order for clinical tests to be valid it is imperative that the disc be of good quality. The Food and Drug Administration tests determine the quality of the disc. The method used is a performance test, where the disc is placed directly on a plate inoculated with a standardized test organism. On the same plate are placed standard (control) discs of five different concentrations. Three plates are used and six discs of each batch are tested. The potency is determined by measuring the zones of inhibition, plotting a standard curve, and reading from the curve the antibiotic concentration of the average zone size obtained for the sample. The uniformity is determined from the range of zone sizes obtained among the six sample discs. The requirement established by experimental data is that the maximum difference in zone diameter between any two of the six zones of inhibition be no greater than 2.5 mm. The 95% confidence limit of the potency test is 67 to 150%. These limits were established by extensive experimental data. Many of the assays for specific antibiotics have narrower intervals, but rather than setting a different one for each antibiotic we set the interval that all of them could meet. Therefore we have suggested this range as the tolerance for the labeled potency of sensitivity discs.

To have a standardized clinical test all discs of the same labeled potency must perform the same. In order to accomplish this, commercial discs, in their preparation, must be standardized against control discs. The proposed Antibiotic Regulations give explicit instructions for the preparation of the control discs which are used for the standard curves. In addition to specifying the preparation of standard antibiotic solutions they specify the diameter and grade of paper to be used for the preparation of the control discs. Some questions have been raised about the reproducibility of results obtained with the paper grade we have specified. We recently completed a detailed study to investigate this question. Four different batches of paper of the same grade manufactured over a period of two years were obtained. Four different antibiotics were impregnated on each of the four different batches, respectively. Tests were performed with these discs over a period of several days and 1,152 zones of inhibition obtained. A statistical analysis showed no significant difference among zone sizes obtained with the four different batches of paper.

Conversely, however, when control discs were prepared using different grades of paper significantly different results were obtained among the different grades. By batches we mean lots of paper of the same grade, as specified by the Food and Drug Administration, manufactured at different times. By different grades of paper we mean paper of different specifications.

In conclusion, it is felt that an official form of control of antibiotic discs is desirable. First of all, standardizing both labeled and actual potencies results in a better understanding of the use of the disc by the medical profession. Secondly, and most important, the high-quality standardized disc allows a more accurate test in the clinical laboratory with the resulting benefits to the patient.

COMMENTS BY CHAIRMAN

DR. LOUIS GREENBERG: Thank you, Dr. Kirshbaum. We faced similar problems in Canada a few years ago. In our case our government was very kind to us. Our Justice Department ruled that, in accordance with our Food and Drugs Act, sensitivity discs could be classified either as a "device" or as a "drug." For administrative purposes it was decided to include discs as drugs and in practice we control them as we do other drugs.

AN EVALUATION OF ANTIMICROBIAL SUSCEPTIBILITY TESTING

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A year ago at the Symposium on Antibiotics reference was made to the questionable quality of susceptibility testing that was being done in some of the routine diagnostic laboratories in the United States [1]. In an effort to correct this apparently serious situation recommendations have been made to standardize susceptibility testing on a national and international basis [1, 2]. Apparently there has been some progress in this direction. An expert committee was convened by the Director General of the World Health Organization in Geneva this past summer to make recommendations for a standard testing method. The program for disc certification initiated three years ago by the Food and Drug Administration is nearing fruition [3]. In Canada certification of disc content has been in effect [4]. These efforts are significant and, it is hoped, are stepping stones to the eventual standardization of susceptibility testing. However, certification that discs will not contain less than nor more than a certain percent of the labeled content, certification with respect to an expiration date, and pointed information relative to the care and storage of discs, can all go for naught if the user does not pay attention to the above information, does not intelligently apply the test, and does not know how to correctly interpret the results.

The crux of the whole situation is apparently to get everyone to do susceptibility tests by a method or methods which, when interpreted, will not only mean the same thing but will carry to the physician intelligent, understandable information and furnish him with a better therapeutic guide.

We hear so much about variables that reportedly enter into our routine susceptibility test methods that it is no wonder that some physicians say susceptibility tests are meaningless. Are there so many variables in the routine test that they cannot be overcome? If so, then reliability will never be placed on susceptibility testing and it would perhaps be better, as has been suggested, not to do any. From my experience I do not believe that that day will ever arrive. I do not believe the results of an in vitro susceptibility test will ever be an exact duplication of what is going to occur in the body. I do believe that, through some national or international organization, standards of methodology for the in vitro susceptibility testing of microorganisms to therapeutic antimicrobial agents could be and should be formulated. Then we could intelligently exchange results; we would have a truer evaluation of the development of resistance in microbial species; we would have a true meaning of the terms "susceptible" and "resistance"; we would have a defined understandable interpretation of the vague term "moderately susceptible" or "moderately resistant"; we would reduce the oft-spoken-of variables in the test methods to a minimum; we would give insurance to the physician that in so far as an in vitro test dictates, the results have meaning; we would be affording a needed service to the patient.

I wish to return for a moment to the term "moderately resistant". Just what does that mean? Does it mean that half the cells in a test culture population are inhibited? Three quarters of the cells? Undoubtedly it means that it takes more of the same agent to inhibit strain A of the same species than strain B or one microorganism versus another. If that is true then the physician should be alerted to use more of the agent if it is the one of choice. I think, if it is permissible and ethical, that the term "moderately resistant" should be qualified by the statement, "Susceptible only to a greater concentration of the agent than that achieved with the usual dosage. If this agent is used an increased dosage is suggested."

In keeping with this line of thought, how often is an organism that is isolated from a urine specimen reported resistant when by virtue of the increased concentration of the agent in the urine it could be really susceptible? I appreciate that this concept does not apply to all disease conditions of the urinary tract, for example, prostatitis. However, where an organism isolated from urine is found to be resistant to the concentrations of antimicrobial generally used in the routine test, a retest with a higher concentration of the agent could elicit significant information in this specific instance. It would seem advisable, therefore, to use higher test concentrations of the antimicrobials when determining the susceptibility of organisms isolated from the urine than are used in testing organisms isolated from other areas of the body. This is possibly being done; if not, it should be. In line with this thinking it might be well, if not too complicated, to adopt a protocol of susceptibility reporting similar to that used in Sweden, and grade the organism relative to susceptibility as 1, 2, or 3, etc. This I have referred to before [1]. The susceptibility-testing method of choice in a routine diagnostic laboratory depends upon several factors. The disc test which is adaptive to the volume of work and the available help, has been the one most widely used. The broth test-tube method, though more accurate than the disc method, is more of a special than a routine test, though I understand some laboratories have high and low concentrations of antimicrobials tubed in broth or agar that are used in a routine manner [5]. Some laboratories prefer to use the agar-well, gradient-plate, plate-dilution, plate-dilution with replicating apparatus, or the hemoglobin-reduction method [6, 10]. Comparative studies of some of these methods with the disc and test-tube methods have been done [11, 15]. It would appear that any of these methods, when competently applied and interpreted, has a place equal with the others. However, each of these methods requires more manipulation than the disc method. One could assume that methods such as the gradient-plate or plate-dilution method, in which a measured amount of antimicrobial is incorporated in the medium, could give a more quantitative result than the disc method. The rapid hemoglobin-reduction and the agar-well methods are essentially modifications of the disc test. The so-called variables associated with the disc method are then, in part, inherent in these two modifications.

Since it is common practice to compare all other methods with the tube-dilution method one may assume that this is a standard method for susceptibility testing at the present time [16]. I believe it is. I believe also that a test medium or media for use with the test-tube method should be agreed upon. I say media because some fastidious species will not grow on all media. Possibly a general medium for most species and a special medium for the more fastidious species would be an answer. I believe also that a standard inoculum in a range of from 100,000 to 500,000 cells/ml would represent a good cross section of the test population. Studies that we have made show no significant differences in the end-point of susceptibility by the test-tube method in the above range except for penicillinase producing staphylococci when tested against penicillin. The concentration of staphylococci in a test tube susceptibility test should not be less than 500,000 cells/ml.

Tryptone broth, among others, is satisfactory in the in vitro testing of sulfonamides with a broth test-tube method. This medium exhibits less inhibitory effects on sulfonamides than do some more complex media. However, tryptone will not support the growth of the more fastidious organisms. In my opinion in vitro testing of susceptibility with the sulfonamides is of little value. It would seem more correct to judge the effectiveness of sulfonamide activity on a clinical basis with supporting cultural studies.

The in vitro testing of the susceptibility of Mycobacterium tuberculosis to the antituberculosis agents is in such a specialized area of testing that it is not commonly determined by a general routine laboratory. The standardized test-tube method as described appears to be satisfactory in our experience [17]. Discs for this susceptibility test are available using Peizer's medium [18]. I am not in a position to discuss the adequacy of this disc test.

The susceptibility testing of Neisseria gonorrhoeae is probably not done in most laboratories. This is possibly because it has been stated that this organism is so susceptible that testing is not usually necessary [19]. It may be that such an inference was in part a precipitating factor to the present staphylococcal problem. On the contrary, it is often necessary to determine the susceptibility of N. gonorrhoeae, especially to penicillin, and a quantitative test should be done. Since the disc test with penicillin is too qualitative for this determination and no satisfactory test-tube method has been developed, the agar plate-dilution method should be used with a chocolate agar or equivalent medium. The reason for accurate susceptibility testing of N. gonorrhoeae is not that strains are becoming alarmingly resistant to penicillin. They are not. But strains are being found that are less susceptible to penicillin. In general most strains of N. gonorrhoeae are inhibited in vitro by concentrations of penicillin in a range of from 0.002 unit to 0.2 units. However, strains of N. gonorrhoeae being found to require 0.3 to 0.5 units of penicillin for in vitro inhibition have raised the need for accurate susceptibility testing. To the best of my knowledge, the disc method is insensitive to this degree of difference in susceptibility. Patients infected with strains susceptible only to the larger increments of penicillin require a different dosage regimen to assure bacteriologic cure.

The agar-diffusion-disc susceptibility testing method has been criticized as unreliable because of the many variables in the test. Factors most commonly referred to as inducing variability are the medium used, inoculum size, pH, depth of agar, period of incubation, moisture, and other physical factors.

There have been reports in the literature of media interfering with the end results when some antibiotics have been tested by the disc method [20]. Though I have observed some differences in endpoints with different media these have not, in my estimation, been of the order that would make a susceptible organism resistant or vice versa. However, if definite media were adopted that would most closely simulate conditions under which the antimicrobial would be active in vivo contradiction and disagreement in results done by different laboratories, would be minimized. There are several basic media in common use for routine culturing, to which blood may be added, that will not introduce any significant variation in the disc test. To compare susceptibility on EMB and on blood agar is not valid. Most laboratories will use, within a few milliliters, the same amount of medium in their blood-agar plates. It takes gross differences in the depth of agar in a petri plate to introduce a significant difference in the disc-test result.

The pH of the medium most widely used is in the range from 6.8 to 7.2. In that area there would be no gross error introduced into the result. Tests should be run in a pH range that simulates conditions in vivo. It might be advisable to test urine isolates in a slightly more acid medium.

The amount of moisture in the medium has been considered to introduce error. I have purposely applied saline and distilled water to dry discs to see what would happen. There was no significant difference in the zone size with the addition of moisture.

The inoculum size is most frequently mentioned as an important variable in disc susceptibility testing. A heavy inoculum for the disc test, as I have stated relative to the tube-dilution test, is significant in representing a good cross section of the population. In the disc test, growth on the plate should be confluent. This can be achieved by using a 4.0 mm loopful of a 24-hr broth culture. The cell density of this inoculum will approximate one to ten million cells, depending upon the species. Inoculum size produces the most significant variation when penicillinase producing staphylococci are tested with penicillin. An inadequate inoculum will result in erroneous reports of penicillin susceptibility with penicillinase producing strains of this genus regardless of the method.

The period of incubation before reading susceptibility tests has been referred to as inducing variable results. An 18-24 hr incubation period most aptly fits into the working program of most routine diagnostic laboratories. Studies that I have done with the disc and test-tube dilution methods show no significant differences in endpoints in readings made at 8, 18, or 24 hr. In my opinion an 18-24 hr reading may be more valid than a reading made in a shorter period of time.

These so-called variables, and others that have been mentioned, in my opinion, are minor problems that could be corrected with the development and acceptance of standardized methods for susceptibility testing. I believe the main problems lie not in the tests per se but in the dissemination of and intelligent adherence to proper instructions on how to perform and how to interpret susceptibility tests, with special reference to the disc agar diffusion method. The disc test has a little more to it than just inoculating a plate, putting a paper disc impregnated with therapeutic antimicrobial on the plate, looking for a zone, and saying "susceptible" or "resistant".

A method of disc susceptibility testing using discs of a low and high, or of low, high, and intermediate potency has been advocated [19]. A disc of each potency is placed on the inoculated plate. After incubation the results are interpreted by the presence or absence of a zone. A zone around both low and high discs, regardless of diameter, is interpreted as susceptible. A zone around only the high disc means moderately resistant. No zone around each disc means resistant. Instructions accompanying commercially available discs direct the user to the two or three disc method and its interpretation. I agree only with the last interpretation, that no disc zone implies resistance. I cannot agree with the statement that, and I quote, "The presence of a zone of inhibition indicates susceptibility of the organism to that concentration of the therapeutic agent being tested while the absence of a zone indicates resistance." [21]; or, and I quote again, "The presence of a zone of inhibition, regardless of size, around low, medium and high concentration discs indicates the organism is very sensitive to the therapeutic agent method, and would have a low minimum inhibitive concentration (MIC) by the tube dilution method" [22].

I have been using a low potency disc method for the past twelve years [1]. There are many strains of organisms that are resistant, although zones 10 mm in diameter or less are quite evident. The only exception to this interpretation is as I have previously stated [1]. One cannot, in my opinion, interpret disc susceptibility tests without measuring the zone size and using a guide of a previously established comparison with tube-dilution susceptibilities. By this I do not mean that it is correct to interpret the susceptibility of an organism by comparing the zone size of two different therapeutic antimicrobial agents. These zone diameters are not necessarily going to be the same, even if the organism is susceptible to both agents. It is as false to say that the diameter of the zone should be the same regardless of the agent when an organism is susceptible, as it is to say that an organism is susceptible to 2 or 30 μg of an antimicrobial agent, when tested with discs of that potency, because there is a zone of inhibition. Strange as it may seem, this is being done, Directions accompanying discs, as I have pointed out, indicate that results may be interpreted in this manner. Unless these concepts are corrected, and unless the disc test is not considered to be just a crude qualitative test, this method of susceptibility testing might well be abandoned because it may be doing more harm than good. The disc test, however, when properly done, is a valuable guide to our present day therapy. I refer all who are interested in susceptibility testing to the excellent article by Ericsson [23], which appeared last year in Antibiotica et Chemotherapia, and from which I quote: "Dilution methods using liquid medium are generally considered to be superior to diffusion methods with solid medium. In this paper it is shown that culture on solid medium is probably preferable from the biologic point of view, and also that diffusion methods using antibiotic-impregnated discs can, with meticulous technique and observation of certain sources of error, give quantitative results of fully satisfactory accuracy."

I have already referred to and described elsewhere a method for determining the susceptibility of microorganisms using a single low potency disc of each antibiotic [1]. Interpretation is not based on the mere presence or absence of a zone of inhibition, nor based directly on the potency of the disc. Interpretation is made in accordance with a calibrated guide prepared by comparative studies of zone diameter with the tube-dilution susceptibility. There is also the single disc method of Kirby, et al. [24] in which a single high potency disc is used. Interpretation by this method is also in terms of the diameter of the zone in accordance with a guide calibrated to the high potency disc. Each of these single disc methods is basically a standardized procedure the details of which can be found elsewhere [1, 24].

SUMMARY

It has not been the purpose of this report to compare the available methods for susceptibility testing one with the other.

The purpose has been to point out areas in our present methodology where certain changes are necessary in order to improve the quality of susceptibility testing especially as it is done by the disc agar-diffusion method.

To make these changes requires cooperation. It is hoped that this can be found and with combined effort there will arise a standard method or standard methods for susceptibility testing that will be acceptable nationally and internationally.

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AN ATTEMPT TO INTEGRATE THE RELIABILITY OF SUSCEPTIBILITY TESTING WITH CLINICAL RESPONSE

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I appreciate the opportunity of participating in this panel on certain aspects of clinical laboratory results in the use of antibiotics. I have been more fortunate than many laboratory workers in being closely associated and enjoying the cooperation of practicing clinicians, especially in the field of urinary tract infections. Our efforts to determine what is best for the patient have resulted in several cooperative investigations of antibiotics and their use.

I wish to preface my remarks with the following three statements which served as a guide when experience was limited and still serve as a guidepost with accumulated experience.

The efficacy of any chemotherapeutic agent is a function of the patient's tolerance. Expressed in another way, the patient must tolerate the chemotherapeutic agent before it can be evaluated.

Each patient should be considered as an individual or a 100% statistic in determining whether or not an antibiotic should be used. There have been more occasions than not when antibiotic therapy has been contraindicated, in spite of laboratory data on an organism's antibiotic susceptibility.

We should not expect a 100% correlation between in vitro and clinical response. Any current in vitro method employed to ascertain susceptibility or resistance to an antibiotic and the translation of its meaning to clinical application is generally qualitative.

This is all well and good, but from a practical standpoint it is expected that a routine qualitative testing procedure of bacterial susceptibility will be followed in a hospital or clinical laboratory. On that basis we ask ourselves how reliable that "lab report" is.

To obtain maximum correlation between clinical response and in vitro susceptibility results, we tested discs of each of the antibiotics under consideration by the agar diffusion technique. Standardization of the in vitro testing was determined by comparison with the minimum inhibition concentration of the serial tube-dilution and plate-dilution techniques, and by correlation with clinical response. We shall discuss only the agar diffusion-disc technique. Two basic considerations should be stated here. First, those organisms primarily isolated in urinary tract infections, such as Staphylococcus aureus (coagulase-positive or coagulase-negative), enterococci, Escherichia coli, Aerobacter aerogenes, Pseudomonas aeruginosa and Proteus species, were consistently definitely resistant or susceptible by the criteria used. It was rare to encounter a questionable reaction. Secondly, no proven incidence of change in resistance in vitro during therapy has been noted, but there is evidence of removing the susceptible strains and permitting the resistant varieties to emerge.

Penicillin, streptomycin, chlortetracycline (Aureomycin), oxytetracycline (Terramycin), and chloramphenicol (Chloromycetin) have been used against the above-mentioned organisms since 1951; nitrofurantoin (furadantin) since 1953; tetracycline and erythromycin since 1954; sulfisoxazole (gantrisin), novobiocin, and oleandomycin since 1956; and kanamycin (Kantrex) since 1958. The organism isolated was considered susceptible if it showed a 2-mm zone of inhibition around a 2-unit disc of penicillin, 10 μg of streptomycin, 30 μg of chlortetracycline, oxytetracycline, or tetracycline, 10 μg of chloramphenicol, 2 μg of erythromycin or oleandomycin, 15 μg of novobiocin, 300 μg of sulfisoxazole, or 30 μg of kanamycin; or a 5-mm zone around a 100-μg disc of nitrofurantoin. Although tests were made against streptomycin from 1953 to 1956, they were not routinely continued beyond that period because this agent is rarely used by the physician except in some specialized cases, such as tuberculosis. Streptomycin is therefore omitted from this discussion. With respect to neomycin, bacitracin, and polymyxin, our experience has been limited and our data are scanty; therefore, these three antibiotics do not enter into this discussion although it is well known that they are good agents when indicated. Enough of what we did not do; let us discuss what we did do!

There have been more than 20,000 examinations made for bacterial susceptibility on approximately 4000 microorganisms, using the conditions cited. Considerations for the final evaluation were concentration of organisms, time and temperature of incubation, and antibiotic concentration, as well as disc-types.

We employed the following criteria to determine clinical response: Cured indicated complete elimination of symptoms and pyuria, whether or not the urine became sterile; improved indicated marked reduction, but not elimination, of both symptoms and pyuria, whether or not the urine was sterile; and unimproved indicated no reduction of either symptoms or pyuria and usually also the persistence of the microorganism.

We could not evaluate clinical correlation with laboratory results in approximately one case in five in which in vitro susceptibility tests were made. This was due to incomplete data because of inadequate follow-up, inadequate laboratory tests, and interuption of treatment because of serious side effects. In vitro susceptibility tests were made on the isolated organisms and the clinical course of the cases were followed carefully. At this time no attempt is made to include patients with mixed infections or those with a questionable clinical diagnosis.

In no way does Table 1 indicate the relative efficacy of the antibiotics used. The figures are influenced by the fact that a relatively large number of resistant Proteus strains were included in the nitrofurantoin study. Only Gram-positive organisms (mostly sensitive) were included in the oleandomycin study. Primarily organisms sensitive in vitro were included in the chlortetracycline study and one

Table 1. In Vitro Susceptibility Testing

Antibiotic	Total number of cultures	Number susceptible	Number resistant
Nitrofurantoin	41	16	25
Oleandomycin	32	27	5
Sulfisoxasole	36	13	23
Novobiocin	35	24	11
Tetracycline, three forms	138	90	48
Oxytetracycline	51	39	12
Chlortetracycline	50	40	10
Kanamycin	23	19	4
Total	406	268	138

Table 2. Correlation Between the Susceptibility of an Organism and Favorable Clinical Response

Antibiotic	Number of patients with susceptible cultures	Patients cured or improved		Patients unimproved	
		Number	%	Number	%
Nitrofurantoin	16	12	75	4	25
Oleandomycin	27	25	93	2	7
Sulfisoxasole	13	11	85	2	15
Novobiocin	24	24	100	0	0
Tetracycline	90	74	82	16	18
Oxytetracycline	39	35	90	4	10
Chlortetracycline	40	31	77	9	23
Kanamycin	19	17	89	2	11
Total	268	229	Avg. 85	39	Avg. 15

of the tetracycline studies. The prime factor determining the selection of these cases was the availability of complete clinical data as well as laboratory data. In general, it is natural to expect this table to show a higher than usual percentage of susceptible strains since one generally does not set an antibiotic regimen of therapy when the organism is resistant.

With this background, let us see what degree of correlation we obtained between in vitro and clinical response. Tables 2 and 3 summarize this data.

Table 2 indicates the correlation between susceptibility of organism and favorable clinical response. When patients demonstrating organisms susceptible in vitro to an specific antimicrobial agent, were treated with agent, favorable clinical response was between 75% and 100%, with an average of 85%.

When patients demonstrating organisms resistant in vitro to a specific agent, were treated with that agent, unfavorable clinical response was between 0% and 64%, with an average of 34% (Table 3).

Table 3. Correlation Between Resistance of an Organism and Unfavorable Clinical Response

Antibiotic	Number of patients with resistant cultures	Patients unimproved		Patients cured or improved	
		Number	%	Number	%
Nitrofurantoin	25	8	32	17	68
Oleandomycin	5	2	40	3	60
Sulfisoxazole	23	9	39	14	61
Novobiocin	11	7	64	4	36
Tetracycline	48	12	25	36	75
Oxytetracycline	12	4	33	8	67
Chlortetracycline	10	5	50	5	50
Kanamycin	4	0	0	4	100
Total	138	47	Avg. 34	91	Avg. 66

In summary, we see that we can expect, within the limits of this testing method, from 75% to 100% favorable response in organisms susceptible in vitro, or approximately 15% showing an unfavorable clinical response. However, there has been a consistently high incidence of favorable clinical response in contradiction to the indicated in vitro resistance of the organism. We find that approximately two out of three organisms, resistant in vitro, will give a favorable clinical response. Immediately one begins to question the criteria as expressed.

As one may see, we are not concerned with increasing our incidence of indicated susceptible strains. The correlation between in vitro susceptibility and favorable clinical response of from 75% to 100% is surprisingly good. What we are concerned about is the relatively poor correlation between incidence of in vitro resistant organisms and the lack of clinical improvement. Here two factors may be operating. First, the clinical criteria may be too lax, indicating a higher favorable response than may actually have resulted from the specific therapy. The other factor concerns the question of reliability of manufacturer's claim of the stability of the amount of antibiotic in the disc. We might also question whether the 66% who responded favorably in spite of an indicated in vitro resistance would not have improved spontaneously with no antimicrobial treatment.

PANEL DISCUSSION ON CLINICAL LABORATORY TESTING

DISCUSSION

MR. COWLES: I am very much interested in the control of sensitivity discs; that's my job and I was very much interested in Mr. Kirshbaum's and Dr. Gerwe's comments. Mr. Kirshbaum says that in 1957 this program with the Food and Drug Administration was undertaken, that there was a definite need for it, and there is no question that there has been a tremendous improvement in the quality of sensitivity discs since that time. The Food and Drug Administration has standardized methods and enabled laboratories to follow some uniform procedure.

I was also interested in Dr. Gerwe's comments, the fact that the U.S.P. Committee on Scope was considering the possibility of having discs come under U.S.P. regulations. Over the past three years, there has been an opportunity to work out some of these problems between industry and the Food and Drug Administration, but on December 31 the certification of discs becomes the law of the land, subject to all laws of enforcement and all the penalties that are available under the Food, Drug, and Cosmetic Act.

Now, my point in bringing this whole thing up is this. The test that we have essentially depends upon the control discs. The description of control tests is given in close detail in the Food and Drug regulations. But the fact remains that, no matter who follows these regulations and how carefully they are followed, there is going to be a difference in the way the "control" discs are made. I am very much concerned about this point, and I believe it should be given serious thought because, as I said, when it becomes the law of the land it will be enforceable with all the penalties of the law. At the present time we prepare our own control discs as a matter of expediency. I well realize the problems of preparing "control" discs for general distribution. They present an almost insurmountable problem. But this method of expediency may get us into trouble. Unquestionably, if it ever goes into the United States Pharmacopoeia, reference control discs would be necessary for the Pharmacopoeia would never accept the procedure that is used at the present time by the Food and Drug Administration.

I bring this question up now because it's a difficult problem to answer. Possibly somebody in this audience or someone concerned with the problem can come up with some thoughts on this subject. I am seriously concerned about it, for if it does become law how can one enforce it without reference control discs being available for the control tests. That's the thought I wanted to leave with you.

MR. KIRSHBAUM: The main objection I think Mr. Cowles has raised was the necessity for reference discs to be distributed by somebody. We have thought a great deal about this. However, if this is done, the body that is going to distribute them would in turn become manufacturers of discs. The number of discs that would be needed for daily use by all disc manufacturers in the establishment of standard curves would be more than any one manufacturer is making now. We think it's impractical, but we do think that the control disc can be made.

DR. SCHERR: I would like to comment on the matter of control discs and then raise one other small point because Dr. Gerwe mentioned the need of control discs, too. I hope Mr. Kirshbaum will bear me out if I recall that the figure of 67-150% of labeled potency was arrived at by taking a series of paper discs and carefully pipetting standardized solutions of antibiotic on to them. By doing an assay on the amount of antibiotic in the disc, it was found that the best you could do by this method was 67-150% of the labeled potency. How can one manufacture and standardize commercial discs to be between 67-150% of the labeled potency if the accuracy of the reference disc, which is literally tailor-made, varies to this same degree. Unless this particular problem is resolved and other criteria of standardization are made, you are going around in a circle because the standard discs will have the same deviations from labeled potency as is demanded of the manufactured disc.

The second point I wish to raise is this: in about two months, if and when this certification program is instrumented, one of the things that will happen is that the profession at large will accept the fact that a great step forward has been made and a great reliability has been placed on sensitivity disc testing as a whole. If you examine the regulations carefully, they say that only seven antibiotics are certified and I think I can mention them. They are streptomycin, dihydrostreptomycin, tetracycline, chlortetracycline, penicillin, chloramphenicol, and bacitracin. No other antibiotic or no other antimicrobial agent is certifiable. This is not the situation in Canada. If you put an antimicrobial agent on a sensitivity disc and you send it into Canada, you are subject to the control of the Canadian authorities in Ottawa.

However, if you should package any other sensitivity disc other than these seven, in the same package with these seven, they automatically become subject to certification. Now, the question I wish to pose is: What is the rationale of requiring that a sensitivity disc impregnated, let's say, with novobiocin be subject to certification if it is shipped out in a common package with a disc containing penicillin when it is completely immune to certification if it is shipped out in a separate package? If this were understood it would take a tremendous amount away from the reliability of the entire certification program. These seven certifiable antibiotics represent only a small fraction of the number of antimicrobial agents presently put into discs.

MR. KIRSHBAUM: In regard to the first point that Dr. Scherr raised. It is true that the confidence limits of 67-150% were arrived by discs that we prepared ourselves. However, this a rather broad confidence spread; it covers all eventualities. If, for example, we tested penicillin discs we would prepare both the "standard" discs and the unknowns. The limits expressed refer to the entire assay and not to an individual disc. If we have a perfect standard, an absolute standard which, of course, is not available, the confidence limits might have been narrower.

The second question is a good one. I personally agree with Dr. Scherr. It does put us in somewhat of an embarrassing position to say that novobiocin discs will not be certified and penicillin discs will be certified. But, this is one of the shortcomings of democracy. The power to certify discs which was invested in the Food and Drug Administration covered only the seven certifiable antibiotics. We do not have the authority, which must come from Congress, to certify the other antibiotics, so we are doing the best we can with the law.

DR. GREENBERG: In connection with standard discs, we have made a great number of discs by methods similar to that advocated by Mr. Kirshbaum's laboratory and have assayed them carefully by extraction techniques. I doubt very much if the variation between discs when prepared in this manner would be any more than plus or minus 10%, and even that would be high.

MR. FOWLER: I wonder if we are missing the point. We have been interested in this disc test for a long time and we have been interested in the upgrading of discs. We knew about them when they were not performing well. Our studies today indicate that these discs are of high quality. We have purchased some on a number of occasions, tested them, and found them measuring up to quality standards when used within the expiration date. That is not the problem.

The problem is interpretation. This is the thing that has to be upgraded today. The manufacturers have upgraded their products; they have done their part. Now, it's up to the hospitals to upgrade the interpretation. At the present time, all kinds of interpretations are being used, Dr. Kirby, the F.D.A., the manufacturers, and that group I spoke about this morning are all using different criteria. They are so widely apart that it is like saying that we have a 3-ft pole here, we have a 5-ft pole here, and an 8-ft pole over here. Those people who say they can get uniform results by doing their test are saying that a 3-ft pole equals an 8-ft pole. This is not true. Dr. Kirby's standard does not equal the F.D.A.'s, and they do not equal that of the other group. What we need is a standard to which we can release any method. That standard is lacking.

DR. VERA (Baltimore Biological Laboratory): I am concerned with control work on the media used in the tests. I disagree with Mr. Fowler and some of the other people as to a standard method. There are so many variables concerned with the test that it would be impossible to establish a single standard method. Recommended procedures for different types of cultures or different types of specimens could be developed. If the case is acute or if the laboratory does not know whether it is acute or not, the work should be done as if the case were a very serious one. There is no reason why one procedure cannot be more efficient for urgent cases than another procedure. It is not necessary to start with a low concentration of disc only if you need important information about the case. On the other hand there isn't any reason why one could not start with a low disc, if the cases are more or less chronic in nature, and the test is being done as a control of therapy, rather than on an initial isolation. In such cases all sorts of controls can be established, even tube-dilution controls. But a standard procedure would not, and could not, take into consideration expedient factors, and, after all, a laboratory is trying to find susceptibility.

Yesterday, in the discussion, Mr. Kirshbaum mentioned the fact that there is a very important difference between bacteriostatic agents and bactericidal agents. A drug that is commonly called bactericidal is not necessarily bactericidal for everything that is applied, even to strains of the same species. It is very easy to fail to detect susceptibility by failing to observe the cultures when they are growing; by waiting longer, because it's more convenient or because the laboratory doesn't function on Sundays, a zone can well be missed. It has been our experience in the last year or two, that we have had many more complaints. We are asked why it is that there is clinical response but no detection of susceptibility? In some cases there is failure to recognize a bacteriostatic action and to take proper precautions to check for susceptibility. Sometimes there are failures to use sufficiently dilute cultures. Staphylococcus or Proteus or Pseudomonas, let us say, will give you confluent growth on a plate that is swabbed without any difficulty, but sometimes overnight cultures may have a thousand times that many organisms. You have such a tremendous application of organisms, neutralizers, inhibitors, and what have you, that you will not detect susceptibility, whereas with a simple dilution you will find it.

I think there is less correlation between a therapeutic response and laboratory report of resistance, than there is on the cultures that are reported susceptible and do respond. In our opinion, certainly in our correspondence, that has been true. Some of these failures to detect susceptibility, I'm quite sure, are due to factors that have been discussed here. The discs may be improperly stored, used for an indiscriminate length of time, exposed to heat, moisture, light, etc. With repeated exposure and prolonged exposure, it is a wonder that they work as well as they do. There are other failures, which I consider failures to make the most of the test as it is done. Having had personal experience with control cultures, control work, controlled inoculae, controlled media, I do not see how we can control the reactions of an organism within a millimeter, to specific amounts of drug. And yet some of the recommended procedures are based on requirements within the order of a millimeter difference. If you cannot do it with a well-worked standard culture you cannot expect to do it with a primary culture, or with a fresh isolate. Nor can you decide whether an organism is resistant or nonresistant on the basis of such a small margin. It doesn't seem realistic to me.

Just one other point. Failure to detect susceptibility in some cases may be because the high level discs that we now employ commonly are too low for some organisms. Data on that subject are now being developed. As you know, in Europe the concentrations of drugs are generally much higher than those used in this country and it is true that with massive doses of penicillin there are more responses to Proteus infections, to Streptococcus fecalis infections, and similar chronic diseases. It is possible that sufficient data may develop to show the need for higher potency discs for that sort of work, and, although I rather doubt that we would use it for all sensitivity testing, I think perhaps it should be made available. This could be another reason why the laboratory may fail to detect susceptibility in some cases.

DR. GREENBERG: Dr. Anderson, would you care to comment on the need for high potency discs for the testing of sensitivity in certain infections.

DR. ANDERSON: I suggested that in urinary infections, probably there are areas where we do need a higher concentration disc. I see no reason why you might not use 100 μg discs in testing organisms from urinary tract infections, where you can get extremely high concentrations of the drug with excretion. From personal experience in our diagnostic laboratories, I think such a procedure would be perfectly acceptable. Sensitivity testing is not as difficult or clouded over as we have been led to believe. There is no reason why it can't be realistically standardized to a position where we won't be making errors. As far as missing interpretation in laboratory, I am sure this probably happens, but I think that it's better to err on the side of indicating that an organism is resistant than to try to make an organism susceptible that isn't susceptible. If the organism is susceptible the clinician should have some hope that the drug will be effective. But if you err on the other side, then you are just guessing.

DR. KIRBY: I would like to say that I agree completely with the point that Dr. Anderson has just made, and, in fact, with his whole talk. I really think one of the deficiencies here is that there has not been correlation and publicizing of sensitivity testing by people who know the clinical side and the laboratory side. The main education of the doctor and the laboratory is done by the literature sent out by the people who make the discs and this literature contains the sort of false and fallacious statements cited by Dr. Anderson in his talk. When the disc manufacturers met three or four years ago to talk about standardardization they did a very valuable thing and I think standardization is coming. A great deal has been accomplished in the last three or four years and the people who could make the greatest contribution would be the disc manufacturers, if they could meet with people who understand these things and issue some worthwhile instructions to the laboratory. The information that thousands of laboratories get about performance and interpretation are inadequate.

I think that if one is going to take an attitude that we really know nothing, it's hardly worth doing the test. There are certain of us, such as Dr. Anderson and myself, who have worked with these tests from a clinical and laboratory standpoint for ten years, and who think we have standardized methods that are extremely helpful in guiding therapy. We do not find instances where the organism is resistant, but the patient responds, that we cannot account for. This is a matter of adequate clinical and laboratory correlation and interpretation. So I would simply make this suggestion, that a great deal could be accomplished by some meetings and improvement of the literature that is dispensed with the discs.

DR. GREENBERG: I would like to point out that there are quite a number of hazards attached to the establishment of a general standard procedure. This is particularly true in a test where so much depends on the individual who is doing the testing and the interpreting. Certain aspects of the test might well be standardized but in the long run there will be no substitute for the procedure Dr. Kirby and Dr. Anderson followed when they put in much time and effort correlating their laboratory results to the clinical response. Each laboratory will have to determine this for themselves.

REPORT ON MEETING IN GENEVA WITH WORLD HEALTH ORGANIZATION

DR. WRIGHT (FDA): We were merely at this meeting, as were all others, not as representatives of their particular countries or particular institutions. I was elected Vice-Chairman; it is a purely honorary position, so I don't know why they wanted to honor me, but there it was. As Dr. Branch indicated, the meeting was quite well organized. We had a very long agenda and the chairman, Dr. Welch of Belgium, did conduct the meeting with dispatch.

All phases of antibiotic sensitivity testing were discussed, from the importance of bacterial resistance to the indications for performing individual tests, that is, the reasons for making the determinations. There was a review of all the various methods that are used. We discussed the recommended standard methods, which include the disc method as the recommended method for clinical use, and the dilution method (primarily agar dilution method) for more precise work that would be done in surveys. The report is quite long, and I think that actually it can only be appreciated by reading it in its entirety. I don't think there has been anything that has been mentioned in the discussions today that isn't covered in some way or another in the report, although there are a few things in the report that haven't been mentioned today. Although we knew what was in the report, as we attended the committee meeting, we had no indication when the contents of the report would be cleared for dissemination or for discussion in any public meeting. But Dr. Branch has received communication from Dr. Sensinez, which indicated it is permissible to release it.

I believe that the report will be published in its entirety in the Bulletin of the World Health Organization. The thing that impressed me as one of the most important things in the report is the subject of interpretation of results. Almost everyone this morning and this afternoon, Dr. Fowler, Dr. Anderson, Dr. Kirby, certainly was concerned with the importance of proper interpretation. And there is a section of the report devoted to "Reading of Results". Of course, the results are ones which are obtained using more or less standardized procedures. I think I should probably read it verbatim so that you don't lose its meaning too much. First, inhibitions always should be measured. These measurements can be translated into minimum inhibitory concentrations in micrograms per milliliter. Graphs may be made relating these two values for each antibiotic under the exact conditions of the test. Such data will not be available in many laboratories and, although this is the ideal method of interpretation and reporting, a simple alternative must be permitted. The simple alternative, of course, is not to interpret these zone sizes as other than minimal inhibitory concentrations, but simply as sensitive, moderately resistant, and resistant. However, these interpretations should be made in connection with the experience of the person in the laboratory, the bacteriologist, with strains which are of known susceptibility. The section closes with a very important warning. "In no case, should zone size as such, be reported." Of course, the whole principle of interpretation here, the zone sizes being converted to minimal inhibitory concentrations, or even to various broad classes of susceptibility, is dependent on the standardization of the method, the particular disc or discs which are used, against organisms of known susceptibility. And that's one of the most important recommendations of the committee, namely that strains of agreed-upon standard susceptibility be set up. They felt that, for each antibiotic, three strains would be sufficient to set up a response line which will cover a fairly wide range and will enable the laboratory to convert the zone sizes to MIC, or to some broad classes of susceptibility.

On the storage of the discs, the main warning has to do with what is to be done once the container has been opened. It warns against opening the containers immediately after taking the container from refrigerator. This is a very elementary warning, but I'm sure that very often in the stress of getting things done in a hurry, the bottle may be taken out of the refrigerator, opened, and the discs taken out. In the meantime, condensation has occurred very rapidly and the bottle may be capped up and put back in the refrigerator, but by that time, the damage has been done. The principle of control over the discs was endorsed. The problem of standardized discs was recognized as a problem and, of course, it's not possible to resolve that problem. However, one of the recommendations was that the World Health Organization investigate the possibility of setting up some kind of standard discs which could be distributed. The difficulties to be overcome in distributing and preparing standard discs are not minor ones.

Other than that, I can't say much about the report without going through every section. I would strongly recommend when you get a chance, and when the report is available, that you read it and I think you will appreciate it very much.

I think that being aware of the principal recommendations of the committee would certainly be of use to you in taking some steps toward achieving some standardized approach to the interpretation of the disc results.

EXPERIENCES WITH A CYTOTOXICITY TEST AS A PRIMARY SCREEN FOR DETECTION OF ANTITUMOR AGENTS IN FERMENTATION LIQUORS OF ACTINOMYCETES

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INTRODUCTION

Cytotoxic substances, which are also active against experimental tumors in vivo, can be produced by microorganisms [1, 2]. Dilution and agar-diffusion techniques for in vitro assay of cytotoxic activity of antitumor agents have been described [3, 4]. The availability of a rapid, inexpensive and reproducible screening tool to replace the experimental tumor screen in animals would greatly enhance the effectiveness of the search for agents with potential antitumor activity in human neoplasias. No microbiological system or combination of systems is known which selects, a priori, agents which will prove to be effective against experimental tumors in animals or against human tumors.

The purpose of this investigation was to determine whether potential antitumor agents present in fermentation liquors of actinomycetes could be detected by a preliminary cytotoxicity screen.

METHODS

All of the actinomycete cultures employed were soil isolates. Spores or mycelia from slant cultures were suspended in sterile distilled water and were used to inoculate the fermentation medium. The fermentations were carried out in a variety of nutrient media for 4 days on a reciprocal shaker at 27 C. The fermented media were centrifuged and the clarified fermentation liquors were quick-frozen and stored at −15 C until tested for cytotoxic and antitumor activity.

A total of 693 fermentation liquors was selected on a random basis from our screening program to be tested for cytotoxic activity and for effect on experimental mouse tumors. Forty-six broths which had already passed an experimental mouse-tumor screen were also tested for cytotoxic activity.

The dilution tissue-culture assays employing KB epidermoid carcinoma cells were performed according to the specifications [5] of the Cancer Chemotherapy National Service Center (CCNSC) at the School of Medicine, University of Miami, Coral Gables, Florida, under the direction of Dr. M. M. Sigel. A broth was considered cytotoxic if it gave an ED_{50} at a dilution of 1 : 100 or greater.

Screening of the fermentation broths in mice at the maximum tolerated dose against the solid subcutaneous forms of sarcoma 180 and mammary adenocarcinoma 755 and the ascitic form of lymphoid leukemia L-1210 was also performed according to CCNSC specifications [6] at Microbiological Associates, Inc., Washington, D. C., under the direction of Dr. I. Kline. In all, 436 broths were tested in the sarcoma 180 system only, while 257 broths were tested in all three systems. None of the broths tested exhibited activity in the leukemia L-1210 system. Response in the sarcoma 180 and adenocarcinoma 755 systems was determined by growth inhibition based on weight of the excised tumors in a two-stage test. A sequential analysis technique on a statistical basis passed those materials in which $(T/C)_1 \times (T/C)_2 = <0.20$. Several fermentation samples were depleted in the course of the two-stage test which was then completed on a subsequent sample prepared to duplicate the original one.

Antitumor antibiotics produced by the actinomycetes were characterized on the basis of their antimicrobial spectrum and paper chromatography. Frequently this could be accomplished using the fermentation liquor; in some cases the antibiotic was isolated and purified as an aid in identification.

RESULTS

A total of 693 fermentation liquors was tested for cytotoxic activity and for effect on experimental tumors of mice and the results obtained are presented in Table 1.

Table 1. Cytotoxic and Antitumor Activity of 693 Fermentation Liquors

Number of liquors	Tissue culture activity	Antitumor activity
601	–	–
84	+	–
5 (2+3*)	–	+
3 (2+1*)	+	+

*The two-stage antitumor test was completed on a sample prepared to duplicate the original.

Eighty-four of the 693 broths tested were active against KB cells in tissue culture but failed to inhibit in an experimental tumor system. Six hundred and one broths failed in both screens.

Altogether, eight broths were found to be inhibitory in at least one of the tumor systems. Four broths on which both cytotoxic and tumor testing were completed on the same fermentation sample were active initially in the adenocarcinoma 755 screen. Two of these exhibited cytotoxic activity while two did not. Subsequent attempts to reproduce the antitumor activity with the cytotoxic-negative broths were unsuccessful. One of the cytotoxic-positive broths contained the antibiotic actinoleukin; the other is being studied further.

Table 2. Status and Cytotoxic Activity of 46 Fermentation Liquors Which Passed an In Vivo Tumor Screen

Status	Number of liquors	Tissue culture	
		+	–
Tumor activity not reproducible	22	3	19
Culture duplicated	5	2	3
Known antibiotic activity present:			
actinoleukin	1		1
actinomycin group	4	4	
azaserine-like	1	1	
Of interest and being worked	13	3	10

In the case of four liquors, a stage of the antitumor testing was completed on a subsequent fermentation prepared to duplicate the original one. A cytotoxicity test was not repeated on these samples. Of the three cytotoxic-negative broths, in two (one active in the sarcoma 180 and one in the adenocarcinoma 755 system) the antitumor activity was not reproducible and work on the third (active in the adenocarcinoma 755 system) is incomplete. In the case of the one cytotoxic-positive broth, the activity in the sarcoma 180 system was not reproducible.

The status of broths from 46 cultures which had passed an experimental mouse-tumor screen and which were then submitted for the cytotoxicity test is shown in Table 2. Of these broths, 13 or approximately 30%, also gave evidence of cytotoxic activity. The cytotoxic-positive broths included four containing actinomycins (three active against sarcoma 180, one against adenocarcinoma 755), one containing an azaserine-like antibiotic (active against sarcoma 180), and three of sufficient interest for further work. The cytotoxic-negative broths included one in which actinoleukin (active against sarcoma 180) was present and ten cultures of interest. The 13 cultures of interest are being examined further in an attempt to identify and characterize the antitumor agents.

DISCUSSION

The purpose of this investigation was to determine whether a tissue-culture dilution assay employing KB epidermoid cells could be used to screen fermentation liquors from actinomycetes for potential antitumor substances. Three of the 87 cytotoxic-positive broths not screened previously for antitumor activity showed positive effects on experimental mouse tumors. In addition, 13 of 46 fermentation liquors which gave a positive effect on experimental mouse tumors were subsequently shown to have cytotoxic activity. Among the antitumor antibiotics characterized in these fermentation liquors were actinoleukin, actinomycins, and an azaserine-like agent. Work is continuing on a group of four cultures active in both the cytotoxic and tumor screens in an attempt to characterize the antitumor activities present.

The use of the tissue-culture assay as a primary screen for fermentation liquors with a cytotoxic effect at a dilution of 1 : 100 or greater would have resulted in discarding five cultures with mouse antitumor activity. Nevertheless, if these cytotoxic-negative antitumor-positive cultures were sacrificed, a primary cytotoxicity screen would eliminate the necessity of testing about 88% of the broths in an experimental mouse-tumor system. However, important classes of antitumor agents might be eliminated by this procedure. For example 33, or about 70%, of the 46 cultures with predetermined mouse antitumor activity exhibited no cytotoxic effect and work is continuing with ten of these.

Eight broths of the 693 tested passed the two-stage antitumor test resulting in approximately 1.16% of the liquors tested passing this screen. To a large extent the number of cultures passing this screen will depend on the selection of cultures submitted to the screen. Preliminary elimination of known actinomycetes producing antitumor antibiotics would tend to decrease the numbers passing the screen.

ACKNOWLEDGMENT

Our thanks are due to Dr. M. Misiek of these laboratories for microbiological characterizations of the antibiotics produced and to Mr. H. D. Taylor of the Biochemistry Department for performing the paper chromatography. Arrangements for providing the cytotoxicity and antitumor test facilities were made by the Cancer Chemotherapy National Service Center.

SUMMARY

Fermentation liquors from 693 actinomycete cultures were tested for cytotoxic action against KB epidermoid carcinoma cells in a dilution test and in experimental tumor systems in mice. Three of these broths passed both the cytotoxicity and tumor screens. Actinoleukin was present in the broth from one culture, the antitumor activity was not reproducible with one, and work is continuing with the third culture. Five broths passed only the tumor screen, 84 passed only the cytotoxicity screen, and 601 failed to pass either screen.

Forty-six culture broths which had already passed an in vivo tumor screen were tested for cytotoxic activity. Thirty-three were found to be inactive and 13 were active. Four of the active group were subsequently characterized as actinomycin producers, one produced an azaserine-like antibiotic, and work is continuing on three cultures. Actinoleukin was present in one broth of the cytotoxic-negative group and work is continuing on ten cultures in this group.

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DISCUSSION

DR. CAMIENER (Upjohn Company): I wondered if the cytotoxicity tests were run in the same beer in which the antitumor tests were run?

DR. HEINEMANN: Initially, the cytotoxicity and antitumor testing were done on the same beer except in the case of, I believe, four cultures in which the second phase of the antitumor test was done on a subsequent beer.

ISOLATION AND CHARACTERIZATION OF ACTINOMYCIN P_2

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John L. Smith Memorial for Cancer Research
and
Donald W. Renn
Chas. Pfizer & Co., Inc., Maywood, New Jersey

INTRODUCTION

Ever since its discovery from Streptomyces antibioticus by Waksman and Woodruff, actinomycin has been attracting considerable attention because of its interesting biological properties as well as its unusual chemical aspects. Numerous publications have appeared in the past regarding the isolation of naturally produced as well as artificially induced actinomycins. Isolation of homogeneous actinomycins has been a formidable problem and some brillant work by the German, British, and American workers, by various types of chromatography, has appeared in recent years. Following availability of the homogeneous components and sensitive paper chromatography technique, several attempts have been made at classification of the various actinomycins. The most recent and particularly useful review on this subject has been the paper by Katz and Pugh [1].

Our interest in actinomycin has centered on its antitumor activity. Actinomycins C and D have received some clinical trials in the past few years and efforts in finding other members of this group with more favorable therapeutic indices have continued.

Examination of the products of a newly isolated strain of Streptomyces revealed an actinomycin mixture, a component of which appeared to be somewhat less toxic than actinomycins C and D. Paper chromatography of this component

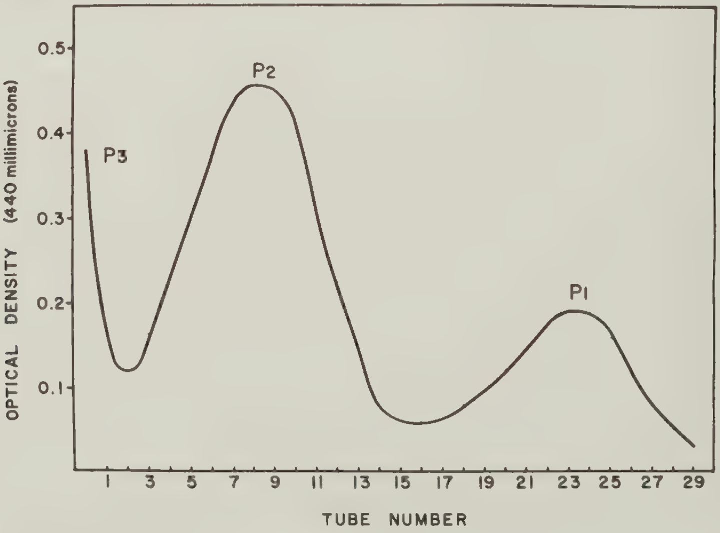

Fig. 1. Countercurrent distribution of actinomycins P_1, P_2, and P_3.

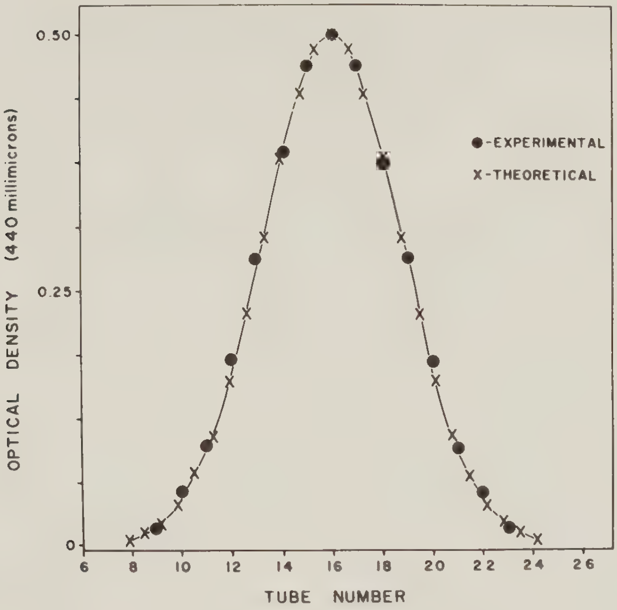

Fig. 2. Countercurrent distribution of actinomycin P_2.

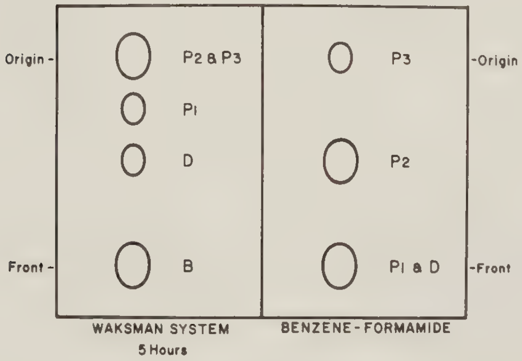
Origin -
P2 & P3
P1
D
Front -
B
P3
-Origin
P2
P1 & D
-Front
WAKSMAN SYSTEM
5 Hours
BENZENE - FORMAMIDE

Fig. 3. Paper chromatography of actinomycin P_2.

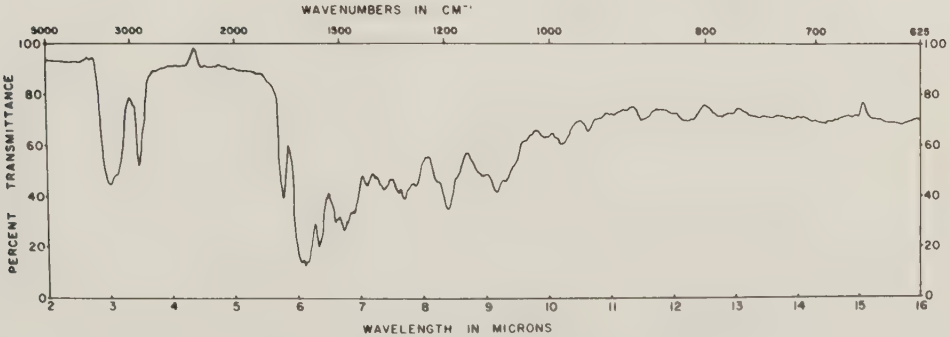

Fig. 4. Infrared spectrum of actinomycin P_2.

also distinguished it clearly from these actinomycins. We obtained more of this material and its isolation and characterization form the subject of this paper.

Fractions from the purification steps were tested by paper chromatography, using two systems. In the system noted by Waksmann [2] the new component did not move. In a benzene—formamide system this component had an Rf value of about 0.6.

The crude actinomycin was isolated by extraction with n-butanol and the product was first subjected to chromatography on alumina in ethyl acetate solution. About 90% of the total actinomycins appeared in the effluent and wash. The chromatography was continued successively with more ethyl acetate followed with 10% methanolic ethyl acetate. The fractions from the last solvent contained most of the component of interest. Paper chromatography of the crystalline material at this stage indicated the presence of three actinomycins which we designated as P_1, P_2, and P_3.

Further purification of actinomycin P_2 was carried out by countercurrent distribution in a pyridine—benzene—ligroin—water system (4 : 4 : 3 : 4). Figure 1 shows the distribution pattern of the three components. After a repetition of the distribution the product was crystallized twice. At this stage actinomycin P_2 appeared homogeneous in countercurrent distribution (Fig. 2) and showed a single spot on paper chromatography in the benzene—formamide system (Fig. 3). Elemental analysis: C, 56.10; H, 7.29; N, 12.37. The infrared spectrum run as a potassium bromide pellet is shown in Fig. 4.

The activity of actinomycin P_2 against some of the Gram-positive bacteria is given in Table 1. A detailed study of the antitumor activity in animals as well as in tissue culture which will be published elsewhere indicated actinomycin P_2

Table 1. Antibacterial Activity of Actinomycin P_2

Microorganism	MIC, μg/ml
Staphylococcus aureus	0.78
Streptococcus pyogenes	0.39
Streptococcus faecalis	1.56
Diplococcus pneumoniae	1.56
Bacillus subtilis	0.78
Lactobacillus casei	0.39
Aerobacter aerogenes	>200
Escherichia coli	>200
Mycobacterium sp. 607	0.39

Table 2. Antitumor Activity of Actinomycin P_2

Test	Dose, mg/kg	Survival rate	Inhibition, %
Sarcoma 180	0.75	5/6	70
	0.60	6/6	61
	0.40	6/6	45
Adenocarcinoma 755	0.45	10/10	94
	0.30	10/10	93
	0.15	10/10	61
HeLa cells in tissue culture	0.05-0.10 μg/ml (Lethal)		

to have a somewhat better therapeutic index than either actinomycin C or D. A brief description of the type of activity against Sarcoma 180 and adenocarcinoma 755 is given in Table 2. Actinomycin P_2 has been in clinical study for some time and preliminary results have been published recently [3].

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. T. M. Lees for paper chromatography, to Dr. A. R. English for the bacterial spectrum, and to Dr. J. J. Oleson for the antitumor data.

SUMMARY

The isolation and characterization of actinomycin P_2, a member of the actinomycin family has been described. Actinomycin P_2 exhibits a somewhat lower toxicity than actinomycins C and D, which are being used in cancer chemotherapy.

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DISCUSSION

DR. FUSARI (Parke Davis): When you gave the countercurrent system what was the ratio of the solvents?

DR. RAO: The ratio was 4:4:3:4.

DR. FUSARI: How about the benzene—formamide system?

DR. RAO: That's merely the paper impregnated with formamide.

DR. KATZ: Have you tested to find what amino acids are present?

DR. RAO: Those are underway. We have at least threonine and hydroxyproline accounted for and there are two more which are not yet thoroughly identified. We had three or four batches of this material from various fermentation sources and they show some differences.

THE PRODUCTION OF TWO STRUCTURALLY UNRELATED ANTITUMOR AGENTS, ACTINOMYCIN AND ANTIMYCIN A BY STREPTOMYCES ANTIBIOTICUS CULTURES*

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INTRODUCTION

In the course of antitumor screening operations in these laboratories, a streptomycete culture was isolated which produced beers having in vivo activity against sarcoma-180,† carcinoma-755, and leukemia-1210 tumors in mice [1]. Subsequent investigation established the presence of an actinomycin in the beers but the in vivo data could not be reconciled with the presence of a single antitumor component. After extraction of the actinomycin, a preparation with in vivo antitumor activity was obtained which showed, by paper chromatogram analysis against KB cells in tissue culture [2], the presence of at least one new (and at that time unidentified) component which previously had been "masked" by the actinomycin [3]. In subsequent purification experiments both components of the beer were crystallized and were identified as an actinomycin F or Z complex and an antimycin A complex. The in vivo antitumor activity of both antibiotics was confirmed in mouse tests and these compounds are currently undergoing extended antitumor evaluation in experimental animals.

The production of these two structurally unrelated antibiotics by a single streptomycete proved interesting since this particular phenomenon had not been reported previously. Taxonomic studies increased interest further since they showed that the isolated organism was a variant of the well-studied species, Streptomyces antibioticus. Subsequent survey studies undertaken as a result of these findings and employing a rapid spectrophotometric assay developed during the latter phases of purification have shown that both antibiotics are produced by S. antibioticus strains in general and by no other streptomycete tested.

MATERIALS AND METHODS

Cultures. The culture used in the majority of experiments presented in this paper was a variant of S. antibioticus (S. sp. 163) isolated in the course of antitumor screening operations. It has been designated UC 2499 and NRRL 2838. The culture is maintained in soil.

Other streptomycetes used included seven S. antibioticus strains obtained from Dr. R. W. Rivett of the University of Wisconsin, the CBS,‡ the NRRL

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*This work was supported in part by Contract SA-43-ph-1933, Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Washington, D. C.

†In vivo antitumor testing was performed at the Armour Research Foundation, Chicago (Dr. Sidney Mittler), at the Sloan-Kettering Institute for Cancer Research (Dr. Christine Reilly), and at the Children's Cancer Research Foundation, Boston (Drs. Charlotte L. Maddock and George E. Foley).

‡CBS, Centraalbureau voor Schimmelcultures, Baarn, Holland; NRRL, Northern Utilization and Research Division of Agricultural Research Service of U.S.D.A., Peoria, Illinois; ATCC, American Type Culture Collection, Washington, D.C.; UC, Upjohn Cultures, Kalamazoo, Michigan.

(No. S-1543, B-1702, and B-1703) and the ATCC (No. 8663 and 10,328); *Streptomyces flaveolus* (CBS); *Streptomyces flavus* (CBS); *Streptomyces chrysomallus* (ATCC 11523); *Streptomyces michiganensis* (NRRL B-1940); *Streptomyces parvullus* (ATCC 12434); *Streptomyces parvus* (ATCC 12433); and six Streptomyces sp. (Upjohn Culture Collection No. UC-2087, UC-2102, UC-2291, UC-2438 and UC-2443, and NRRL 2288). Streptomyces sp. NRRL 2288 is an antimycin A producing culture; all other cultures are actinomycin producers. As with UC-2499 (above), all of these cultures are maintained in soil.

Shake-flask fermentations. Seed and fermentation experiments were carried out in 500-ml wide-mouth Erlenmeyer flasks containing 100-125 ml of media. The flasks were incubated at 28 C on a Gump rotary shaker operating at 250 rpm with a 2.5 in. stroke. Vegetative seed was prepared by inoculating the flasks with a loopful of soil and growing the organisms for 3 days in a medium containing 25 g of Cerelose and 40 g of cottonseed meal per liter of tap water. Fermentation flasks were then inoculated with 1 ml of seed/100 ml of medium and the flasks were incubated for 2-5 days. The composition of the fermentation media are given in Table 1. Medium 1 allows excellent production of both antibiotics; media 2 and 3 favor the formation of antimycin A; medium 4 favors the formation of actinomycin. Maximum antimycin A production is achieved in 2-3 days; maximum actinomycin production occurs in 4-5 days.

Table 1. Fermentation Media

Components, g/liter of tap water	Medium			
	1	2	3	4
Cerelose	20	–	–	–
Glycerol	–	25	25	–
Cottonseed oil	–	–	–	15
Dried yeast (Difco)	2.5	2.5	–	2.5
Defatted soybean meal*	10	10	20	10
Ammonium sulfate	5	5	5	5
Calcium carbonate	4	4	4	4
Potassium chloride	3	3	–	3
Presterilization pH	6.8	6.8	6.8	6.8

*Kay Soy, Archer-Daniels-Midland Company, Chicago, Illinois.

Tank fermentations. Six seed flasks prepared as indicated above were used to inoculate a 100-gal fermentor containing 66 gal of sterile tank seed medium (presterilization pH 7.2) of the following composition: 10 g of Cerelose, 10 g of corn-steep liquor, 2 g of defatted soybean meal, 10 g of Wilson's Peptone 159, and 2 ml of lard oil/liter of tap water. The seed fermentor was incubated for one day at 28 C and aerated at the rate of 3.54 standard ft^3/min. Constant agitation was effected with a 280 rpm impeller. The 100-gal seed fermentor was used to inoculate a 2000-gal tank containing 1300 gal of sterile fermentation media (Table 1) and the fermentation was carried out at 28 C for 3-5 days. The beer was aerated at the rate of 72 standard ft^3/min and agitated by a 166 rpm impeller. At the conclusion of the fermentation, the mycelium was recovered by filtration.

Assay. The initial purification of both antibiotics was followed with a tissue-culture assay [4]. Subsequent purification procedures were based on the spectrophotometric assay presented below.

RESULTS AND DISCUSSION

Spectrophotometric assay for antimycin A and actinomycin. The following assay is based on the purification procedures used to isolate crystalline antimycin A and actinomycin. One hundred milliliters of whole beer at pH 7 were filtered under vacuum through a coarse-grade fritted-glass Büchner funnel and the filter cake was compressed to remove entrained liquid. The volume of clear beer was measured and subtracted from 100 ml to obtain the "volume" of mycelium. The packed mycelium was slurried with four "volumes" of absolute methanol, stirred for 20 min, and refiltered as above. The methanolic extract was then dried by passing a stream of air over the surface of the liquid.

The dried mycelial extract was extracted with 25 ml of Skellysolve B for 10-15 min with occasional agitation and the Skellysolve was decanted into a clean beaker and evaporated. The Skelly-washed residue was then re-extracted with 25 ml of toluene and this solution was also evaporated. Both the evaporated Skellysolve and toluene residues were then dissolved separately in 25 ml of absolute methanol and the samples were examined in the Cary Recording Spectrophotometer from 260 to 400 mμ and from 350 to 550 mμ. Most of the antimycin A was found to be extracted by Skellysolve while the bulk of the actinomycin was found in the toluene extract.

The spectral curves which were obtained are shown in Fig. 1. The ΔOD values at the maxima were obtained by measuring the magnitude of the absorption from the drawn straight lines (see Fig. 1) to the peaks on the curves. Delta OD values were used in subsequent calculations ($\Delta OD_{\lambda} = \Delta a_{\lambda} ct$) to compensate for nonspecific absorption of light at wave lengths at or near the maxima. In some instances high optical densities have been observed at 320 and 450 mμ with OD values approaching zero. Reproducibility of the spectrophotometric assay is excellent. Experimentally, $\Delta a_{450\ m\mu}$ for the actinomycin complex produced by

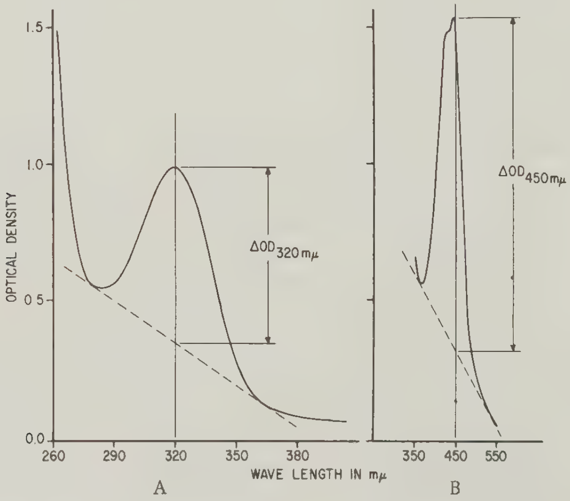

Fig. 1. Representative ultraviolet and visible spectra of partially purified antimycin A and actinomycin. Whole beers were prepared for spectrophotometric examination as discussed in the text and the methanolic solutions were examined in a Cary Recording Spectrophotometer. Straight (dotted) lines were drawn on the curves as shown in the figure. The ΔOD values are calculated by measuring the absorption between the straight lines and the maxima. A) Ultraviolet spectrum of partially purified antimycin A complex in methanol. This particular sample was prepared from a three-day fermentation of UC-2499 in Medium 1. B) Visible spectrum of partially purified actinomycin F or Z complex in methanol. This sample was prepared from the same shake-flask fermentation used in Fig. 1A.

Table 2. Appearance of Actinomycin-Producing Cultures on Ektachrome [11]

Organism	Bennett's	Czapek's sucrose	Maltose tryptone	Peptone iron	0.1% tyrosine	Casein starch
Streptomyces sp. 163 UC 2499	Trace gray aerial Yellow-tan reverse	Trace gray aerial Gray reverse	Gray aerial Brown reverse	No aerial Brown reverse H_2S darkening	Trace gray aerial Red-tan reverse	Trace gray aerial Yellow-tan reverse
S. antibioticus Rivett	Fair gray aerial Yellow-tan reverse	Trace gray aerial Gray reverse	Gray aerial Brown reverse	No aerial Brown reverse H_2S darkening	Trace gray aerial Faint pink reverse	Trace gray aerial Yellow-tan reverse
S. antibioticus CBS	Fair gray aerial Yellow-tan reverse	Trace gray aerial Gray reverse	Gray aerial Brown reverse	No aerial Brown reverse H_2S darkening	Trace gray aerial Red-tan reverse	Trace gray aerial Yellow-tan reverse
S. antibioticus NRRL S-1543	Fair gray aerial Yellow-tan reverse	Trace gray aerial Gray reverse	Gray aerial Brown reverse	No aerial Brown reverse H_2S darkening	Trace gray aerial Dark red reverse	Trace gray aerial Yellow-tan reverse
S. antibioticus NRRL B-1702	Fair gray aerial Yellow-tan reverse	Trace gray aerial Gray reverse	Gray aerial Brown reverse	No aerial Brown reverse H_2S darkening	Trace gray aerial Red-tan reverse	Trace gray aerial Yellow-tan reverse
S. antibioticus NRRL B-1703	Trace gray aerial Tan reverse	Trace gray aerial Gray reverse	Gray-white aerial Brown reverse	No aerial Brown reverse H_2S darkening	Trace gray aerial Faint pink reverse	Trace gray aerial Yellow-tan reverse
S. antibioticus ATCC 8663	Fair gray aerial Yellow-tan reverse	Trace gray aerial Gray reverse	Gray aerial Brown reverse	No aerial Brown reverse H_2S darkening	Trace gray aerial Pink-tan reverse	Trace gray aerial Yellow-tan reverse
S. antibioticus ATCC 10382	Fair gray aerial Yellow-tan reverse	Trace gray aerial Gray reverse	Gray aerial Brown reverse	No aerial Brown reverse H_2S darkening	Trace gray aerial Pink-tan reverse	Trace gray aerial Yellow-tan reverse

UC 2499 was found to be approximately 18 ΔOD units/cm mg/ml; $\Delta\ a_{320}\ m\mu$ for actimycin A equals approximately 8.9 ΔOD units/cm mg/ml.

Isolation and identification of antimycin A complex. Whole beer from a 3-day fermentation (medium 2) was slurried with 8% Dicalite 4200, adjusted to pH7, and filtered. The clear beer containing about 10% of the antimycin activity was discarded and the mycelial cake was eluted several times with approximately 0.5-volume aliquots of methanol. Eluates 3, 4, and 5 were pooled and concentrated in vacuo to an aqueous solution which was then extracted twice with 0.5 volume aliquots of ethyl acetate at pH 5.5. The ethyl acetate extracts were concentrated in vacuo to dryness and the residue was then extracted twice with hot Skellysolve B (ca. 30 ml/g). Concentration of the Skellysolve B extracts gave colorless, needle-like crystals which were further purified by carbon (1 g/g) decolorization

Table 3. The Utilization of Carbon Compounds in Synthetic Medium by UC2499 and S. antibioticus Strains*

Medium	UC2499	S. antibioticus strains						
		Rivett	CBS	NRRL S-1543	NRRL B-1702	NRRL B-1703	ATCC 8663	ATCC 10382
Basal medium	–†	–	–	–	–	–	–	–
+ D-Xylose	(+)	(+)	(+)	(+)	(+)	+	(+)	+
+ L-Arabinose	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
+ Rhamnose	+	+	+	+	+	(+)	+	+
+ D-Fructose	+	+	+	+	+	+	+	+
+ D-Galactose	+	+	+	+	(+)	+	(+)	+
+ D-Glucose	+	+	+	+	(+)	+	(+)	+
+ D-Mannose	+	+	+	+	(+)	+	+	+
+ Maltose	+	+	+	(+)	+	+	+	+
+ Sucrose	–	–	–	–	–	(–)	–	(–)
+ Lactose	+	+	+	+	(+)	(+)	+	+
+ Cellobiose	+	+	+	+	(+)	(+)	(+)	+
+ Raffinose	–	–	–	–	–	–	–	(–)
+ Dextrin	+	+	+	+	+	+	+	+
+ Inulin	–	–	–	–	–	–	(–)	(–)
+ Soluble starch	+	+	+	+	+	(+)	+	+
+ Glycerol	+	+	+	+	–	+	+	+
+ Dulcitol	–	(–)	(–)	–	–	–	(–)	(–)
+ D-Mannitol	+	+	+	+	(+)	+	(+)	+
+ D-Sorbitol	–	(–)	(–)	–	(–)	(–)	(–)	(–)
+ DL-Inositol	+	+	+	(+)	(+)	+	+	+
+ Salicin	(+)	(–)	(–)	(–)	(–)	(–)	(+)	(+)
+ Phenol	–	–	–	–	–	–	–	–
+ Cresol	–	–	–	–	–	–	–	–
+ Na-Formate	–	(–)	–	–	–	–	(–)	(–)
+ Na-Oxalate	–	–	–	–	–	–	–	(–)
+ Na-Tartrate	–	–	–	–	–	–	–	(–)
+ Na-Salicylate	–	–	–	–	–	+	–	–
+ Na-Acetate	(+)	(+)	(+)	(+)	(+)	+	(+)	(+)
+ Na-Citrate	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
+ Na-Succinate	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)

*According to the method of Pridham and Gottlieb (12).

†+, growth and positive utilization; (+), slight growth, possible utilization; (-) poor growth, no utilization; –, no growth.

in ethanol, repeated recrystallizations from methylene chloride-Skellysolve B mixtures (0.25 v/v), and countercurrent distribution. The purified crystalline material had physical and chemical properties (infrared and ultraviolet spectra, specific rotation, solubility behavior, pK_a values, equivalent weight, molecular weight, melting point, and analytical data) identical to those reported for the antimycin A complex [5,6,7].

Isolation and identification of actinomycin. Whole beer from a 4- to 5-day fermentation (Media 1 or 4) was processed as described above for the isolation of antimycin. Following the Skellysolve B extraction of the ethyl acetate residue to remove antimycin, the residue was re-extracted with approximately 25 ml of hot acetone/g of solids and the acetone solution was treated with carbon (0.1 g/g of solids). After removal of the carbon by filtration, the acetone solution was concentrated to about one tenth of the original volume, cooled to 2 C, and the actinomycin crystals were collected. The actinomycin was further purified by repeated recrystallizations from acetone—water and ethanol—water mixtures. The actinomycin can also be obtained from the filtered beer by repeated extractions with ethyl acetate after adjusting the beer pH to 5.5. Paper chromatographic analysis as well as countercurrent distribution studies have shown the presence of at least four components, all of which exhibited maxima in the ultraviolet and visible regions of the spectrum characteristic of actinomycin F or Z complexes [8,9].

Taxonomy. Ektachrome photographs of UC 2499 were compared with Ektachromes obtained from several S. antibioticus cultures and the data are presented in Table 2. The carbon assimilation patterns of these streptomycetes can be found in Table 3. Morphological type comparisons showed that all of the organisms were Type RF [10].* On the basis of the data presented, the culture appears similar to S. antibioticus on Ektachromes and in morphological type and it differs only slightly from S. antibioticus in its carbon assimilation pattern. Thus, UC-2499 has been classified as a variant of S. antibioticus.

Strain studies. Since taxonomic studies had indicated that UC-2499 was a variant of S. antibioticus, it became desirable to know whether the simultaneous and independent production of antimycin A and actinomycin was limited to UC-2499, to S. antibioticus strains only, or to actinomycin-producing streptomycetes in general. Consequently, UC-2499, seven S. antibioticus strains, 11 actinomycin-producing streptomycetes, and one antimycin A-producing streptomycete were tested for their ability to produce antimycin A and/or actinomycin. Fermentations were carried out for 3-4 days on media 1, 3, and 4 and the whole beers were processed for spectrophotometric assay. The results of these experiments are presented in Table 4.

As can be seen from the data, the ability to produce both antibiotics appears to be limited to the various strains of S. antibioticus with only one exception, NRRL B-1703. No other streptomycete tested appears to have this capability when fermented under the experimental conditions employed in this study.

Although the production of both actinomycin and antimycin A by S. antibioticus has not been reported previously as such, there is a possibility that Waksman's colorless "Actinomycin B" material was antimycin A. This supposition is based primarily on the facts that (a) S. antibioticus does produce large amounts of antimycin A, (b) Waksman could have obtained antimycin A with his extraction procedures, (c) the colorless material isolated by Waksman had solubility properties similar to antimycin A, and (d) the relative rates of

*RF, Rectus—flexibilis

Table 4. A Strain Study of the Antimycin A and Actinomycin Producing Capabilities of Several Streptomycetes*

Organism	Antimycin A produced	Actinomycin produced
Streptomyces sp. 163 UC 2499	+	+
S. antibioticus Rivett	+	+
S. antibioticus CBS	Poor growth	
S. antibioticus NRRL S-1543	+	+
S. antibioticus NRRL B-1702	+	+
S. antibioticus NRRL B-1703	+	−
S. antibioticus ATCC 8663	+	+
S. antibioticus ATCC 10382	+	+
S. michiganensis NRRL B-1940	−	+
S. flaveolus CBS	−	−
S. flavus CBS	−	−
S. chrysomallus ATCC 11523	−	+
S. parvullus ATCC 12434	−	+
S. parvus ATCC 12433	Poor growth	
Streptomyces sp. UC 2087	−	+
Streptomyces sp. UC 2102	−	−
Streptomyces sp. UC 2291	−	+
Streptomyces sp. UC 2438	−	+
Streptomyces sp. UC 2443	Poor growth	
Streptomyces sp. NRRL 2288	+	−

*Fermentations and assays were performed as discussed in the text.

production and destruction of both antibiotics noted by Waksman empirically agree with our findings [13,14].

ACKNOWLEDGMENTS

The authors would like to acknowledge gratefully the collaboration of Dr. Joseph E. Grady and Mr. Jeronimo Visser and the technical assistance of Mrs. Nancy E. Lummis and Messrs. Donald R. Horsfall, William L. Lummis, Karl J. Geipel, Darrell K. Edwards, and George C. Prescott.

SUMMARY

The novel and hitherto unreported production of both antimycin A and actinomycin by pure cultures of *Streptomyces antibioticus* was studied. Both antibiotics were discovered through antitumor beer screening coupled with chromatography, purified to the crystalline state by the use of tissue culture and spectrophotometric assay procedures, and identified by a variety of physical determinations. Both antibiotic complexes have in vivo antitumor activity in mice and are currently undergoing extended antitumor evaluation.

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DISCUSSION

DR. KAUFMAN: May I add to the discussion of your fine paper that my colleague, Dr. Schmidt-Kastner was able to produce from Streptomyces ambrozis one type of antimycin and a new type of actinomycin that he called actinomycin U. I think it has not been published yet but he made a patent application in Germany in 1957.

DR. CAMIENER: I had not been aware of this; it is interesting. Was it antimycin A?

DR. KAUFMAN: I don't know which antimycin it was.

DR. CAMIENER: The reason I mention this is because there is some confusion in the literature. There is an antimycin that is produced by a penicillium that is in no way related to Strong's antimycin A material.

DR. KAUFMAN: This antimycin is called filomycin and it is different from the hitherto known antimycins.

DR. REILLY: I wonder, Dr. Katz, if you know anything about it. Would you care to comment on the possibility of the original actinomycin B having been antimycin?

DR. KATZ: There's not much I can add. One point I would make is that in the original isolation of the Streptomyces antibioticus strains, the technique which was used involved E. coli but Streptomyces antibioticus never showed any activity against E. coli.

DR. CAMIENER: Our experience with antimycin A is that it is essentially, relatively inactive. The report on actinomycins A and B was originally published in 1941. Later Dr. Waksman evidently became concerned with the slight trace of contamination of the B with component actinomycin A. He then published another paper in which he brought this point out and suggested that perhaps we ought to re-examine this. In the latest paper on this subject, which I think was in 1954, it was concluded that actinomycin B indeed was a different antibiotic and that some of the data that had previously been reported was the result of its own activity plus that of the contaminating actinomycin. I think that this is where it stands now. There wasn't a sufficient amount of information in the early 1941 paper on the second antibiotic. The paper was primarily concerned with the actinomycin at that time so we don't have as much data today as we might want in order to make an adequate comparison. Part of the difficulty in this area is that the actinomycin is such a very highly active material that it masks the presence of other antibiotics. In many screening operations, for example, a culture that is known to produce actinomycin is discarded and it's only by having some kind of assay, such as a tissue-culture assay in which you can run chromotographic samples, that you can identify components other than actinomycin.

DR. REILLY: Dr. Camiener, maybe I missed something but could you be more specific about the kind of antitumor activity antimycin A has? It's my recollection that we ran Dr. Strong's antimycin A through an antitumor screen and it was not very active.

DR. CAMIENER: In your test, your screen is primarily sarcoma 180 solid tumor. Against this tumor antimycin A was as a plus-minus to a minus, depending on how carefully it was titrated. Two days ago, we got a report from your laboratories and antimycin A had a whole range of activities against other tumors. For example, sarcoma 180 in the ascitic form was very sensitive. This is what our statement of antitumor activity is mainly based on.

METABOLIC EFFECTS OF ORGANIC SULFUR COMPOUNDS ON NEUROSPORA*

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A paper about the use of Neurospora crassa as a primary screening organism of potential antineoplastic agents was presented previously to this society at its sixteenth general meeting held last year [1]. In it, reference was made to some earlier work on a large group of compounds most of which were derived from 4-aminopyrazolo(3,4-d)pyrimidine [2-4].

Several factors were responsible for directing the interest of the authors toward a group of compounds derived from thiophene. One of these factors was the extensive program of syntheses conducted in the Chemistry Department at Texas Woman's University [5-7]. Other factors were the reports in literature about various biological activities of these compounds. Biesele and Jacquez [8] reported polyploidization and multinucleation of sarcoma cells in C-57 black mice with the formation of so-called giant cells brought about by beta-(2-thienyl)-DL-alanine, which is a substitute thiophene. Profft [9] reported that some derivatives of thiophene have anaesthetic effects on the peripheral nervous system. He also observed that some of these thiophenes were bacteriostatic while others were bactericidal.

Of some 90 thiophene derivatives and related sulfur-containing organic substances synthesized, 60 were tested for biological activity with N. crassa Em5256A and some against tumors in C-57 black mice. Some of the thiophenes would cause paralysis and death when injected into mice. This appeared to be due to lysis of the erythrocytes in the animals as was also demonstrated in vitro. The extremities of the animals would turn distinctively red after injection of 4, 5, 6, 7-tetrahydrothianaphthene-4-one and the animals would either die or recover depending on the dosage of the compound injected.

When some of the thiophenes were tested against some adenocarcinomas of C-57 black mice an increase of survival time for some of the injected animals was noted. A slowing down of tumor growth was apparent while injections were given. Although these tests were not discouraging, so far no thiophene tested on tumors has proven to be of more than passing interest.

A comparison of relative activity of the sulfur-containing compounds with some of the antineoplastic agents such as 6-diazo-5-oxonorleucine, amethopterin, nitrogen mustard, and others was made in liquid culture. These data were reported earlier [1]. At the same time studies of some of these inhibitors were presented and references to studies on solid medium with 4-(2-thienyl)butanoic acid were made [10].

Table 1 shows the inhibition of N. crassa Em5256A in 25 ml of liquid minimal medium. The inhibitors are arranged in order of inhibiting capacity with respect to the amount of the compound required to result in 25 or 50% normal dry mycelial weight. In order to compensate for structural differences the third

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*Synthesis supported by the Robert A. Welch Foundation of Houston, Texas. Other support by the National Institutes of Health, Research Grant CY-3853(C3).

Table 1. Inhibition of *Neurospora crassa* Em5256A in 25 ml Liquid Minimal Medium

Inhibitor	ml of compound required to give % inhibition as compared to normal weight		μl of compound in 25 mmoles required to give % inhibition	
	50%	25%	50%	25%
Thianaphthene	0.00020	0.00010	0.68	0.34
2-Benzylthiophene	0.00025	0.00013	1.09	0.57
2-Heptylthiophene	0.00036	0.00017	1.65	0.77
2-Pentylthiophene	0.00068	0.00035	2.61	1.30
2-(2-Methylpropyl)thiophene	0.00085	0.00039	4.10	1.91
4,5,6,7-Tetrahydrothianaphthene	0.00010	0.00050	3.80	1.72
2-(2-Ethylhexyl)thiophene	0.00115	0.00051	5.64	2.05
2-(4-Methylpentyl)thiophene	0.0012	0.0005	5.00	2.10
4-(2-Thienyl)butanoic acid	0.0015	0.0008	6.30	3.40
Thiophenes:				
2-Propyl-5-butyl	0.0016	0.0002	7.30	0.91
2-Nonyl	0.0016	0.0006	8.01	3.00
2-Propyl-5(2-methyl)	0.0016	0.0007	7.29	3.20
2-(2,2-Dimethylpropyl)	0.0029	0.002	11.3	7.8
2-Hexyl	0.003	0.002	12.6	8.4
2-Propyl-2-hexyl	0.004	0.001	21.1	5.3
2-Ethyl-5-hexyl	0.004	0.002	19.6	9.8
2-Ethyl-5-(2-methylpropyl)	0.005	0.003	20.9	12.0
2-Propyl-5-pentyl*	0.005	0.003	24.5	14.7
2-Ethyl-5-(3-methylbutyl)	0.005	0.003	22.6	13.5
2-Propyl-5-(3-methylbutyl)	0.006	0.003	29.0	14.6
2,5-Di-(2-methylpropyl)	0.007	0.003	32.9	14.0
2-Ethyl-5-butyl	0.008	0.004	34.0	17.0
2-(3-Methylbutyl)	0.008	0.004	30.8	15.0
2-Ethyl-5-propyl	0.015	0.008	58.0	30.8
2-Ethyl-5-heptyl	0.017	0.008	89.0	42.0
2-(2-Ethylbutyl)	0.015	0.009	63.1	38.0
2-Nonyl-5-nonanoyl	0.029	0.007	245.0	61.3
2-(2-Methylpropyl)	0.038	0.012	133.0	42.0
2-Undecyl	0.081	0.043	482.0	260.0
2-Allyl	0.100	0.003	290.0	8.6
2-Propyl	0.103	0.006	320.0	18.9
2,5-Di-(3-methylbutyl)	N.R.†	0.008	N.R.	45.0
2-Butyl	N.R.	0.015	N.R.	53.0
2-Decyl	N.R.	0.021	N.R.	118.0
2-Hexyl-5-heptyl	No inhibition			

*Only 91% pure.
†Not reached.

and fourth columns indicate the microliters of compound required to give 50 and 25% inhibition in 25 mmoles of medium.

Each value in the table constitutes the results of several individual analyses, and is an average obtained by mathematical computation. Compounds giving erratic results and those run too infrequently to yield a reliable degree of inhibition were not included in the table. Compounds appearing in the upper portion of the table may be considered good inhibitors, while those in the lower part are weak inhibitors at best. Other compounds that showed no inhibition at all were not included in the table.

Several compounds have shown the ability to colonize or restrict Neurospora growth. Experiments conducted by Tatum et al. [11] revealed that some surface-active anionic compounds, such as tergitol-7 or sodium desoxycholate, caused colonial growth in Neurospora and in Syncephalastrum. There seemed to be no direct relation between the surface activity and the colonial paramorphogenic activity in any of the organisms tested.

During the last two years a large number of chemical compounds was tested in the authors' laboratory for the induction of colonial growth in wild type Neurospora. Two morphological phenomena resulting in colonial growth were easily observable. The first was curling of the mycelia in clockwise direction, sometimes in tight ringlets. The second was bursting and instant coagulation of the discharged protoplasm.

L-sorbose, which is used in inducing colonial growth during mutation experiments, thus avoiding the tedious picking of ascospores, not only slows down growth but permits the hyphal tips to grow in normal fashion with neither curling nor protoplasmic discharge.

Colonial growth in Neurospora was induced by p-hydroxybenzophenone azine, which resulted in an erratic growth pattern of the colonies at the end of 72 hr. The diameters ranged from 5 to 10 mm. Larger colonies with distinct aerial mycelia were produced by 6,8-diamino-2,3-diisopropylquinoxaline. Tight ringlets resulted after 48 hr when Duponol, a surface active agent, was used to colonize Neurospora.

On medium adjusted to pH 3 colonization of Neurospora constitutes the normal growth pattern.

It was theorized that if L-sorbose caused colonization, a large amount of sucrose added to the medium might do so. The normal amount of sucrose in the medium is 2%. An experiment was planned and carried out in which 4, 10, and 20% sucrose was added to the medium. There was considerable restriction of growth at all three concentrations. The 4% concentration restricted growth of the colony diameters to 5-7 mm. There was also some curling of the mycelia. Neurospora subjected to 10% sucrose measured 3-5 mm in diameter. There was curling of the mycelia with exception of 1 or 2 hyphae in each colony which always grew outward in normal fashion. The diameter was the same for both the 20 and 10% concentrations, the difference being that in the 20% concentration the mycelia were in tight ringlets with no wayward hyphae showing. In all cases observed the curling of the mycelia was in the clockwise direction.

Table 2 shows the growth of Neurospora on minimal medium to which different carbohydrates have been added. The highest concentrations of carbohydrates tested which resulted in colonization induction for L-sorbose and inositol were 1 and 0.5%; 0.25% of these compounds would not induce the effect. In another study, without sucrose as carbon source in the medium, these two carbohydrates still colonized. In addition, the same effect resulted with melibiose and with melizitose if tested in the absence of sucrose.

Growth results obtained with ethyl butylthiodimethylethanoate are recorded in Table 3. The last column indicates the number of large and small colonies. It may be observed that down to a concentration of 0.6 ml of a 1 : 200 dilution of the compound only small colonies were observable. At the 0.4 ml level only large colonies resulted. When growth became measurable no colonies were seen since a fine layer of growth had formed all over the surface of the medium.

Table 2. Growth of *Neurospora crassa* Em5256A on Carbohydrates Added to 25 ml Minimal Medium in 125-ml Erlenmeyer Flasks

Carbohydrate	Dry mycelial weight, mg, after 65 hr growth on different percentages of carbohydrate/flask				
	1%	0.5%	0.25%	0.125%	0.0625%
l-Sorbose	16*	19*	36	60	58
Inositol	7*	14*	34	65	68
Melibiose	0	41	58	65	65
Melizitose	19	37	60	56	63
d-Sorbitol	30	68	86	64	64
Control (sucrose)	72				

*Colonizes

The following substances were also tested and found not to inhibit or colonize: l-arabinose, dulcitol, maltose, d-mannose, d-galactose, d-xylose, salicin, lactose, glucose, rhamnose, trehalose, d-ribose, and cellobiose.

Actually, the results as shown in Table 3 are not typical for all compounds tested. In most cases growth and colonization overlapped considerably as can be seen from Table 4. In 1 : 300 dilution, 2-(2-ethylhexyl)thiophene induced colony formation of Neurospora when 1.0 ml to 0.4 ml were added to the medium. However, growth measured 7 mg as dry mycelial weight for 0.8 ml, 21 mg at 0.6 ml, and 34 mg at 0.4 ml, which is almost half the maximum growth of the organism.

The somewhat erratic results between colony formation and dry-mycelial-weight measurements obtained with 4.10-dithiatridecane cannot be explained at this time. These results, however, were consistent in different experiments.

Thiophenes that were found to induce distinct colonial growth in Neurospora were: 1-(2-thienyl)-4-thiaheptane, 1-(2-thienyl)-6-methyl-4-thiaheptane, heptylthiophene, 2-(2-ethylhexyl)thiophene, 2-(2-methylpropyl)thiophene, and ethyl benzylthiodimethylethanoate. The double sulfur compound 4,10-dithiatridecane, not a thiophene, resulted in colonization of the mold in very dilute concentrations covering a wide range of dilutions in which it was active; it proved to be the best colonizer of all compounds tested in this capacity. It was still active in a 1 : 1000 dilution.

These studies on Neurospora show that some of the thiophenes and other sulfur-containing compounds inhibit the growth of Neurospora. They may be con-

Table 3. The Effect of Ethyl Butylthiodimethylethanoate (1 : 200) on *Neurospora crassa* Em5256A

Concentration, ml/flask	Dry mycelial weight, mg		Number of colonies	
	Large colony	Small colony	Large colony	Small colony
1.0	Trace	Trace	0-14	0-8
0.8	Trace	Trace	0-12	0-10
0.6	Trace	Trace	0-9	0-10
0.4	Trace	Trace	12-0	8-10
0.2	4	1	None	
0.1	9	9	None	
Control	80	80	None	

Table 4. Dry Mycelial Weights, mg, of *Neurospora crassa* Em5256A Grown for 65 hr at 25 C on 25 ml Minimal Medium in 125-ml Erlenmeyer Flasks

Compound	Concentration of dilution tested						
	1.0 ml	0.8 ml	0.6 ml	0.4 ml	0.2 ml	0.1 ml	0 ml
2-(2-Ethylhexyl) thiophene 1:300	1*	7*	21*	34*	58	67	81
2-Heptylthiophene 1:300	1*	2*	3*	3*	17	44	81
2-(2-Methylpropyl) thiophene 1:100	0	1*	11*	13*	15	59	111
1-(2-Thienyl)-6-methyl-4-thiaheptane 1:400	1*	1*	4*	4*	4*	7*	81
4,10-Dithiatridecane 1:400	1	1*	3	2	7*	10*	81
1-(2-Thienyl)-4-thiaheptane 1:100	0	0	3*	3*	2*	2*	111

*Indicates colony formation during growth.

sidered, therefore, to be "neurosporacidal" while some other compounds are colonizers and appear to be "neurosporastatic." Of course some fungistatic compounds become fungicidal in higher concentrations.

Colonial growth in Neurospora can be induced by a great variety of compounds which usually also inhibit the amount of growth of the organism. The reasons for these inhibitions are as manifold as the metabolic pathways affected and the various morphological patterns of the inhibited organism.

Although the data presented did in no way answer questions raised with respect to genetically determined colonial growth, they may lead to new approaches for better understanding of possible causes of colonial growth or paramorphic conditions.

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DISCUSSION

DR. EMMONS: The restriction of growth and the curling of hyphae which Dr. Fuerst mentions are suggestive of the curling factor in griseofulvin but this is not a specific response of fungi and probably has no significance. Do you wish to comment on that, Dr. Fuerst? Has this been compared to the type of growth or modification of growth induced by griseofulvin?

DR. FUERST: I have no comparison to make at this time.

IN VITRO ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF SOME 1,3,5-SUBSTITUTED HEXAHYDROPYRIMIDINES

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INTRODUCTION

The high in vitro antimicrobial activity of hexetidine, a 1,3,5-substituted hexahydropyrimidine, has been reported previously [1-10]. Since it is known that the substitution of certain radicals in particular positions may have a marked influence on the antimicrobial activity of a compound [11-16], we undertook the synthesis and subsequent antimicrobial testing of analogues of hexetidine [17,18].

All the compounds studied have a hexahydropyrimidine nucleus with equivalent radicals in the 1- and 3-positions, usually an amino group in the 5A-position, and a hydrogen or alkyl group in the 5B-position (Fig. 1). While the largest series of analogues prepared consisted of 1,3-bis-alkyl-5-amino-5-methyl derivatives, 1,3-bis-aryl- and 1,3-bis-aralkyl- groups were also prepared. In addition, analogues with substitution in the 5A- and 5B-positions were synthesized. The former series included the substitution of a nitro for the amino radical and the formation of various acid derivatives (salts and amides) with the amino radical. In the latter the 5B-position was occupied by hydrogen, methyl, ethyl, or propyl groups. A complete listing of the compounds studied is presented in Table 1.

The method of preparation and the mechanism of the biological activity [2,3, 17,18,19]* as well as additional data concerning the properties of some of these compounds have been published previously [1,4-10]. This paper deals with the correlation of chemical structure with in vitro antimicrobial activity, the action of selected analogues in the presence of chelating agents, and attempts at developing resistance to hexetidine, alone or in combination with other antimicrobials.

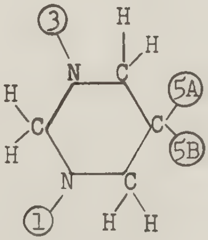

Fig. 1. 1,3,5-substituted hexahydropyrimidine.

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*Guhler, C. J., and H. A. Lardy. Studies of the inhibition of certain enzyme systems in animal tissues by hexetidine, hexahydropyrimidine. Symposium, Northwestern University, October, 1958.

Table. 1. A Series of 1,3,5-Substituted Hexahydropyrimidines Tested for Antimicrobial Activity

Laboratory No.	Bis-1,3-substituents	5 (A) substituents	5 (B) substituents	Salt
L546	–	Methyl	Amino	–
L521	Methyl	"	"	–
L520	Ethyl	"	"	–
L144	Isopropyl	"	"	–
L497	Butyl	"	"	–
L149	1-Methylpropyl	"	"	–
L517	Tert-butyl	"	"	–
L516	Hexyl	"	"	–
L519	1,3-Dimethylbutyl	Ethyl	"	–
W1589	Heptyl	Methyl	"	–
L560	1-Methylhexyl	"	"	–
L499	Octyl	"	"	–
W1571	1-Methylheptyl	"	"	–
L535	Tert-octyl	"	"	–
L325	Decyl	"	"	–
L324	Dodecyl	"	"	–
L326	Tetradecyl	"	"	–
L327	Hexadecyl	"	"	–
L498	3-(2-Hydroxyethylamino)propyl	"	"	–
L145	3-Dibutylamino propyl	"	"	–
L147	2-hydroxy-1,1-dimethylethyl	"	"	–
L557	Phenyl	"	"	–
L148	Benzyl	"	"	–
L524	α-Methylbenzyl	"	"	–
L525	Phenethyl	"	"	–
L407	p-Sulfamoylphenyl	"	"	–
L323	Cyclohexyl	"	"	–
L461	2-Cyclohexylcyclohexyl	"	"	–
L536	Isopropyl	"	Nitro	–
L348	Dodecyl	"	"	–
L558	m-Tolyl	Ethyl	Amino	–
L460	Cyclohexyl	"	"	–
L559	Phenyl	"	"	–
L561	Cyclohexyl	Propyl	"	–
L532	Isopropyl	Hydroxymethyl	"	–
L506	3-Hydroxy-1,1-dimethylpropyl	Methyl	Caproylamino	–
L507	Benzyl	"	Lauroylamino	–
L503	Isopropyl	"	Myristoylamino	–
L501	"	"	Caproylamino	–
L502	"	"	Lauroylamino	–
L504	1-Methylpropyl	"	Caproylamino	–
W1570	"	"	Myristoylamino	–
L549	1-Methylheptyl	"	Palmitoylamino	–
L547	"	"	Lauroylamino	–
L500	"	"	Stearoylamino	–
L556	2-Ethylhexyl	"	Amino	–
L531	"	Ethyl	"	–
W1435	"	Methyl	"	–
L571	"	"	Nitro	–

Table 1 cont.

Laboratory No.	Bis-1,3-substituents	5 (A) substituents	5 (B) substituents	Salt
L538	2-Ethylhexyl	Methyl	Acetamido	–
L539	"	"	Butyrylamino	–
W1573	"	"	Caproylamino	–
W1572	"	"	Isocaproylamino	–
L542	"	"	Heptanamido	–
L544	"	"	Lauroylamino	–
L545	"	"	Myristoylamino	–
L543	"	"	Palmitoylamino	–
L579	"	"	Ureido	–
L573	"	"	Carboxymethyl	–
W1435A	"	"	Amino	Trihydorchloride
W1435B	"	"	"	Monogallate
W1435C	"	"	"	Mono-β-resorcylate
W1435D	"	"	"	Mono-4,4-bis(p-hydroxyphenyl) valerate
W1435E	"	"	"	Monoglutarate
W1435F	"	"	"	Monogentisate
W1435G	"	"	"	Monoacetylsalicylate
W1435H	"	"	"	Monosalicylate
W1435I	"	"	"	Dioxalate
W1791	"	"	Phenylsulfonamide	–

MATERIALS AND METHODS

Antimicrobial activity in vitro. The in vitro sensitivity of bacteria and fungi to the hexetidine compounds was determined by twofold and/or threefold serial dilution in appropriate media.

For the antibacterial testing, dilutions of the test compounds in tryptose phosphate broth were inoculated with 0.1 ml of a 1 : 1000 dilution of an 18-hr broth culture grown at 37 C. The sensitivity of Candida albicans was determined in the same manner using Sabouraud dextrose broth. Trichophyton mentagrophytes was tested in Sabouraud dextrose broth inoculated with a 3.0-mm disc cut with a sterile cork borer from a 10- to 14-day-old culture mat grown on Sabouraud agar at room temperature (22-25 C).

Minimal inhibitory concentrations were determined by visual observation or, in cases of ambiguous end points, by staining and subculture. The antimicrobial activity of the compounds tested is shown in Tables 2 and 3.

Chelating agents. Six chelating agents (diethylenetriaminepentacetic acid, dipicolinic acid, calcium disodium ethylenediaminetetracetic acid, the trisodium salt of N-hydroxyethylenediaminetetracetic acid, and the di- and tetra-sodium salts of ethylenediaminetetracetic acid) were tested in combination with either hexetidine (W1435) or hexetidine trihydrochloride (W1435A) to determine whether they enhanced antimicrobial activity.

The in vitro activity of these chelators against Escherichia coli M51* was determined by the tube-dilution method. Using the same organism, serial dilutions of hexetidine were then tested in the presence of constant subinhibitory

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*Culture from Warner-Lambert stock culture collection. All cultures from this collection are denoted by "M".

Table 2. The Antibacterial Activity of a Series of Substituted Hexahydropyrimidines* as Determined by Serial Dilution Technique

Compound No.	Bacteriostatic concentrations, µg/ml					
	Staphylococcus aureus M16	Klebsiella pneumoniae	Escherichia coli M52	Salmonella anatum	Pseudomonas fluorescens	Proteus sp.
W1589	<1	50	25	12.3	0.45	>400
L560	12.5	44	100	133	133	>400
L499	1	4.9	6.2	14.7	14.7	>400
W1571	1.6	7	55	55	55	500
L325	>100	>100	>100	>100	>100	>400
L324	50	400	400	400	400	>400
L461	50	>400	200	>400	>400	>400
L506	12.5	>100	400	400	400	>400
L507	>400	>100	400	>400	400	>400
L503	4.7	>100	14.8	133	133	>400
L502	2.1	6.3	19	500	166	>500
W1570	2.1	6.3	55	>500	>400	>500
L547	3.7	>100	100	–	>100	>100
L500	<1.5	4.7	50	100	>400	>400
L556	23	6.2	166	500	500	>500
L531	1.5	>100	>100	400	400	>400
W1435	0.5	25	50	400	333	>400
L571	4.9	25	50	400	333	1000
L538	4.9	>100	200	>400	>400	>400
L539	>100	>100	133	>400	>400	>400
W1573	1.6	>100	100	>400	>400	>400
W1572	1.6	>100	133	>400	>400	>400
L542	1.6	>100	200	>400	400	>400
L544	1.6	>50	200	>400	>400	>400
L545	14.8	>50	>400	>400	>400	>400
L543	4.9	>50	>400	>400	>400	>400
L579	3.1	44	200	>400	>400	>400
L573	1.6	400	>400	>400	>400	>400
W1435A	0.7	4.9	50	400	333	1000
W1435B	<0.17	2.6	41	370	740	10,000
W1435C	1.5	13	41	123	1100	1100
W1435D	9.4	28	41	2300	1100	>2300
W1435E	<1.7	1.5	41	1100	1100	3300
W1435F	7	68	41	5000	5000	>5000
W1435G	<0.7	<0.7	184	1666	1666	5000
W1435H	1.2	4.5	123	0,000	1000	5000
W1435I	<0.7	<0.7	20	553	553	5000

*For the structure of the compounds, see the underlined numbers in Table 1.

concentrations of the chelators to determine the relative inhibitory concentrations (Table 4).

The activity of hexetidine trihydrochloride (W1435A) in combination with diethylenetriaminepentacetic acid (DTPA) was determined in a similar manner using five Gram-negative bacteria and three strains of fungi (Table 5).

In vitro resistance studies. In order to determine whether microorganisms develop resistance to hexetidine per se or cross-resistance in the presence of combinations of hexetidine and other antimicrobials several studies were run.

Table 3. The Antifungal Activity of a Series of Substituted Hexahydropyrimidines* as Determined by Serial Dilution Technique

Compound No.	Minimal inhibitory concentrations, μg/ml	
	Candida albicans (10231)	Trichophyton mentagrophytes
W1589	>400	111
L560	200	133
L499	>400	1.6
W1571	15	4.9
L325	133	400
L324	>400	133
L461	25	133
L506	>400	44
L507	>400	44
L503	44.4	44.4
L502	44.4	14.8
L505	14.8	14.8
L547	11.1	11.1
L500	50	4.7
L556	44.4	1.6
L531	>100	14.8
W1435	12.5	4.7
L571	400	14.7
L538	50	14.7
L539	25	14.7
W1573	100	14.7
W1572	44	14.8
L542	>400	14.7
L544	44	4.9
L545	>400	44
L543	200	44
L579	50	4.8
L573	50	14.7
W1435A	37	4.1
W1435B	166	7.5
W1435C	41	4.5
W1435D	85	766
W1435E	41	1.5
W1435F	1660	68
W1435G	185	6.8
W1435H	13	4.5
W1435I	20	0.7

*For the structure of the compounds, see the underlined numbers in Table 1.

In an agar plate dilution of hexetidine using staphylococci, hexetidine (W1435) was incorporated into nutrient agar in exponentially increasing concentrations. The plates were then streaked heavily with 48-hr cultures of coagulase-positive and coagulase-negative staphylococci and incubated at 37 C. Resistant colonies appearing after 48 hr incubation on plates containing inhibitory concentrations of hexetidine were then subcultured in nutrient broth and restreaked on agar containing higher concentrations of the compound. This process was repeated until resistant colonies no longer appeared. In this manner the minimal inhibitory concentrations of W1435 for the parent and mutant strains were determined (Table 6).

In a broth serial dilution of hexetidine using Staphylococcus aureus, hexetidine trihydrochloride and penicillin G were each serially twofold diluted in duplicate in tryptose phosphate broth. Each tube was inoculated with 0.1 ml of a 1:100 dilution of an 18-hr culture of S. aureus ATCC 10055 and incubated for 48 hr at 37 C. The test mixtures containing the highest concentrations of each agent which did not inhibit growth were diluted 1:100 and a 0.1-ml aliquot used as the inoculum for the subsequent dilution series. After the eighth serial passage made in this manner, the development of resistance of the organism to hexetidine trihydrochloride and to penicillin was compared (Table 7).

An agar plate dilution of penicillin and hexetidine using S. aureus was employed to determine the lowest concentration of the test compounds, hexetidine (W1435) and penicillin, capable of producing complete inhibition of visible growth of S. aureus ATCC 10055. Brain-heart infusion broth (Difco) culture, 0.1 ml, containing approximately 10^8 organisms was streaked on series of brain-heart infusion agar plates containing increasing concentrations of the antimicrobial agents. Colonies of bacteria surviving the higher concentrations of the antimicrobial agents after 48 hr incubation at 37 C were counted, survival curves plotted, the organisms subcultured in antimicrobial-free broth for 48 to 72 hr, and the process repeated. To determine the degree of resistance and cross-resistance developed after a series of such transfers, 0.1 ml of a broth culture of the resistant organism was streaked on agar plates containing each of the test antimicrobials (Table 8).

In the final study, S. aureus M4 and E. coli M52 were tested against hexetidine trihydrochloride (W1435A), potassium penicillin G, streptomycin sulfate, polymyxin B, and various combinations of these agents.

Serial passage of the test organism through sets of tubes containing tryptose phosphate broth fortified with graded concentrations of the antimicrobial agent, or agents, was used to determine the original sensitivity of the test strain and to select resistant strains. When combinations of drugs were used in a single test, graded concentrations of one and fixed concentrations of the other were employed.

The degree of resistance and cross-resistance for both the parent strain and the developed resistant strain was determined by assaying (in serial tube dilutions) the sensitivity of each strain against the other antimicrobial agents (Tables 9 and 10).

RESULTS AND DISCUSSION

Antimicrobial activity in vitro. Comparing the static activities of compounds against organisms employed (Tables 2 and 3) with their chemical structures (Table 1), it is apparent that of the 5-amino-5-methyl-5-hexahydropyrimidines, the 1,3-bis-aralkyl derivatives had no significant antimicrobial action whereas the 1,3-bis-alkyl analogues exhibited activity which was clearly defined. This activity first became evident when the carbon chain contained six atoms, reached a maximum in the eight carbon chain compound, and diminished when carbon atoms appeared in excess of eight. Essentially comparable antimicrobial activity was exhibited by compounds with equal numbers of carbon atoms without regard to their arrangement in straight or branched chains.

Table 4. The In Vitro Antibacterial Activity of Hexetidine (W1435) Alone and in Combination with Subinhibitory Concentrations of Five Chelating Agents as Determined by Serial Dilution Technique

Chelating agent	MIC against Escherichia coli M51, μg/ml			
	Chelator alone	W1435 plus μg/ml of chelator		
		0	50	500
Diethylenetriaminepentacetic acid	555	50	3.1	6.2
Dipicolinic acid	>1200	50	12.5	12.5
Calcium disodium EDTA	>1200	50	25.0	12.5
Tetrasodium salt of EDTA	>1200	50	25.0	12.5
Trisodium salt of N-hydroxy-EDTA	>1200	50	50.0	12.5

Table 5. The In Vitro Effect of Diethylenetriaminepentacetic Acid (DTPA) on the Antimicrobial Activity of Hexetidine Trihydrochloride (W1435A)

Compound tested	Proteus sp.	Escherichia coli	Klebsiella pneumoniae	Pseudomonas fluorescens	Salmonella anatum	Candida albicans	Trichophyton mentagrophytes	Aspergillus fumigatus
W1435A	>400	44.4	1.6	400	400	14.8	4.96	4.96
DTPA	185	555	555	1666	1666	20.5	1666	185
W1435A + 100 μg/ml DTPA	<0.06	0.18	0.55	4.9	4.9	<0.06	1.65	<0.06

When the 5-methyl group on an active molecule was replaced by a hydrogen or an ethyl group the activity was not appreciably altered. However, a replacement of the amino group by a nitro group in the 5-position generally appeared to be less effective (in isolated cases, the nitro group increased the effectiveness of the compound against specific strains). Similarly, in cases where the 1,3-bis-alkyl chain contained fewer than seven carbon atoms the addition of alkyl groups to the 5-amino base resulted in greater activity.

Salts of the active preparations seemed to have their own characteristic antimicrobial spectrum, behaving as salts rather than as typical analogues of the parent molecule.

Chelating agents. Of the six chelating agents tested the disodium and tetrasodium salts of ethylenediaminetetracetic acid and the trisodium salt of N-hydroxyethylenediaminetetracetic acid showed some antibacterial activity against S. aureus M16. When tested with various chelating agents the concentrations of hexetidine (W1435) necessary to inhibit E. coli were greatly reduced (Table 4). With the exception of the tetrasodium salt of ethylenediaminetetracetic acid, the addition of 50 μg/ml was sufficient to produce this effect. Diethylenetriaminepentacetic acid (100 μg/ml) greatly enhanced the activity of hexetidine trihydrochloride (W1435A) against five Gram-negative bacteria and three fungi (Table 5).

In vitro resistance studies. The results of the agar plate dilutions of hexetidine using staphylococci show the coagulase-negative strain, Staphylococcus epidermidis M21, to be considerably more sensitive to W1435 than were the coagulase-positive strains. Coagulase-positive strains, S. aureus M3 and S. aureus M21, yielded several mutants showing a twofold increase in resistance but this resistance could not be increased further. No resistant mutants could be developed from one coagulase-negative strain, S. epidermidis M21. S. epidermidis M18 was much more sensitive to W1435 than the other strains tested and showed increased resistance after several passages. However, the resistance developed only to the normal level found in the coagulase-positive strains. After as many as 70 "contact" passages in broth culture, no strain of S. aureus which could grow at concentrations of W1435 higher than 10 μg/ml had been developed (Table 6).

The results of the broth serial dilution of hexetidine using S. aureus (Table 7) indicated that in the eight passages the test organism increased its sensitivity to penicillin from 1.5 to 400-1600 μg/ml but that in a similar eight passages there was no significant change in sensitivity to hexetidine trihydrochloride (W1435A).

Data compiled using the agar plate-dilution method with S. aureus ATCC 10055 (Table 8) indicated that appreciable levels of resistance were developed to penicillin, but that there was no cross-resistance were developed to penicillin, but that there was no cross-resistance to streptomycin or to the hexetidine base there was no development of cross-resistance to streptomycin or to hexetidine base nor was there a reduction in the development of resistance to penicillin.

The comparative broth serial dilution of various antimicrobials indicated that with S. aureus M4, appreciable levels of resistance were developed to penicillin but not to hexetidine trihydrochloride (W1435A) (Table 9). Further, an increase in resistance to penicillin did not result in cross-resistance to this hexetidine compound. In no case did resistance to penicillin result in collateral cross-resistance. With serial exposure to hexetidine trihydrochloride (W1435A)

Table 6. The Minimum Inhibitory Concentrations of Hexetidine(W1435) Required for Parent and Mutant Strains of *Staphylococcus aureus* and *Staphylococcus epidermidis*

Parent strain and resistant derivative	Coagulase activity	MIC of W1435, µg/ml
S. aureus M24	+	4.0
S. aureus M24R		8.0
S. aureus M1	+	4.0
No resistance		
S. epidermidis M21	−	2.0
No resistance		
S. aureus M3	+	4.0
S. aureus M3R		8.0
S. epidermidis M18	−	0.02
S. epidermidis M18R		8.0

Table 7. The Minimum Inhibitory Concentrations (MIC), µg/ml, of Hexetidine Trihydrochloride (W1435A) and of Penicillin Required in Each of Eight Serial Transfers Run in Duplicate on *Staphylococcus aureus*

Drug	Transfer								
	Original	1	2	3	4	5	6	7	8
W1435A	3.1	3.1	1.5	1.5	0.78	3.1	0.78	3.1	3.1
	6.2	3.1	1.5	0.78	0.78	1.5	0.78	3.1	6.2
Penicillin	1.5	>100	200	200	800	400	400	400	400
	3.1	>100	200	400	>800	800	400	>1600	1600

Table 8. In Vitro Sensitiviy and Cross-Resistance of *Staphylococcus aureus* ATCC 10055 to Penicillin, W1435, and Their Combinations (Agar-Dilution Method)

Compound	SP,* µg/ml	IR,† µg/ml	F‡	Cross-resistance					
				Penicillin			W1435A		
				SP, µg/ml	IR, µg/ml	F	SP, µg/ml	IR, µg/ml	F
Penicillin	0.08	100	1250				7.5	7.5	1
Penicillin + 0.3 µg/ml W1435	0.08	80	1000	0.08	80	1000	7.5	5.0	0.6

*SP - In vitro sensitivity of parent strain.

†IR - In vitro sensitivity of induced resistant strain.

‡F - factor of increased resistance: MIC (µg/ml) of induced resistant strain/MIC (µg/ml) of parent strain. Values <1.0 = increased sensitivity; Values >1.0 = increased resistance; W1435 - hexetidine base (aqueous).

Table 9. In Vitro Sensitivity and Cross-Resistance of *Staphylococcus aureus* M4 to Penicillin, W1435A, and Their Combinations (Tube-Dilution Method)

Compound	SP,* µg/ml	IR,† µg/ml	F‡	SP, µg/ml	Cross-resistance				
					Penicillin		W1435A		
					IR, µg/ml	F	SP, µg/ml	IR, µg/ml	F
Penicillin	0.15	154.0	1024.0				2.0	1.0	0.5
W1435A	2.0	7.8	4.0	0.15	0.15	1.0			
Penicillin + 0.2 µg/ ml W1435A	0.07	4.8	68.0	0.15	4.8	32.0	2.0	0.5	0.25
Penicillin + 0.04 µg/ml W1435A	0.15	154.0	1024.0	0.15	154.0	1024.0	2.0	0.5	0.25
W1435A + 0.015 µg/ml penicillin	2.0	3.9	2.0	0.25	0.5	2.0	2.0	3.9	2.0
W1435A + 0.003 µg/ml penicillin	2.0	7.8	4.0	0.25	0.25	1.0	2.0	3.9	2.0

*SP - In vitro sensitivity of parent *Staphylococcus aureus* M4 strain.
†IR - In vitro sensitivity of induced resistant strain.
‡F - Factor of increased resistance: MIC µg/ml of induced resistant strain/MIC µg/ml of parent strain Values < 1.0 = increased sensitivity; Values > 1.0 = increased resistance. W1435A - hexetidine trihydrochloride.

there was no increase or decrease in resistance to penicillin. A delay in the emergence or a depression of the degree of resistance developed to penicillin or both was observed in the presence of some, but not all, subinhibitory concentrations of W1435A. Finally, there was no significant increase in resistance to hexetidine trihydrochloride in the presence of subinhibitory concentrations of penicillin and no development of cross-resistance to penicillin.

E. coli M52 was shown to develop appreciable levels of resistance to streptomycin but not to polymyxin B or to hexetidine trihydrochloride (Table 10). Increased resistance to streptomycin did not ordinarily result in cross-resistance to polymyxin B or hexetidine trihydrochloride nor did exposure to these compounds result in increased resistance to streptomycin. In the presence of a subinhibitory concentration of hexetidine trihydrochloride there was no development of resistance to this hexetidine compound.

The use of combinations of agents to delay emergence and depress the degree of resistance of individual agents in vitro has been studied by many investigators. The use of antibiotics in combination has been recommended as a means of preventing the development of resistant strains of staphylococci and other organisms. Although there is no general agreement that the incidence of resistant strains is reduced by this combination therapy there is definite agreement that such therapy does depress or delay the appearance of resistant strains. Miller and Bohnhoff [20] noted that streptomycin resistance can sometimes be accompanied by an increase in sensitivity to penicillin. Depressing or delaying the appearance of some resistant strains by some antibiotic combinations is probably best illustrated with *Mycobacterium tuberculosis* [21-23]. Patients treated with combinations of streptomycin and isoniazid hydrogine or para-aminosalicylic acid do not develop resistant strains readily, and sometimes not at all. Combinations of erythromycin with either penicillin or streptomycin are also effective in depressing the emergence of resistance in staphylococci. Oc-

Table 10. In Vitro Sensitivity and Cross-Resistance of *E. coli* M52 to Stretomycin, Polymyxin B, W1435A, and Their Combinations (Tube-Dilution Method)

Compound	SP,* μg/ml	IR† μg/ml	F‡	Cross-resistance								
				Streptomycin			Polymyxin			W1435A		
				SP, μg/ml	IR, μg/ml	F	SP, μg/ml	IR, μg/ml	F	SP, μg/ml	IR, μg/ml	F
Streptomycin	7.8	2000	256				0.2	0.2	1.0	25.0	50.0	2.0
	15.6	2000	128				0.2	0.2	0.1	12.5	25.0	2.0
	15.6	2000	128									
	15.6	8000	512									
Polymyxin B	0.2	0.04	0.2	7.8	31.2	4.0				25.0	50.0	2.0
	0.2	0.09	0.5	15.6	31.2	2.0				12.5	100.0	4.0
W1435A	25.0	100.0	4.0	7.8	15.6	2.0	0.2	0.2	1.0			
	12.5	100.0	8.0	15.6	15.6	1.0	0.2	0.8	0.4			

*SP - In vitro sensitivity of parent *E. coli* (M52).
†IR - In vitro sensitivity of induced resistant strain.
‡F - factor of increased resistance: MIC (μg/ml) of induced resistant strain/MIC (μg/ml) of parent strain.
Values <1.0 = increased sensitivity; Values >1.0 = increased resistance; W1435A - hexetidine trihydrochloride.

casionally, however, strains acquire moderate resistance to one or both of these agents while exposed to both. It is generally accepted that an organism must be originally sensitive to both agents of a combination for any delaying or suppressing effect on the emergence of resistance to either agent to occur. The relative doses of antibiotics used in combination therapy are most important for the demonstration of resistance-depression effects.

It is evident from the results of the resistance studies conducted, as well as from the literature, that among the pathogenic microbial organisms each species is unique in its ability to develop resistance and that, within the same species, there exists a potential for the development of strains which may or may not show cross-resistance.

A high degree of resistance to some agents, such as streptomycin, may emerge in a single step. However, our studies bear out the fact (often noted by others) that most organisms usually acquire resistance in stepwise fashion, varying according to the antimicrobial agent and to the bacterial species used and that S. aureus strains herein studied do not develop resistance to hexetidine.

ACKNOWLEDGMENT

The writers are grateful to Dr. Floyd E. Anderson for the preparation of the last nine compounds listed in Table 1, and to the late Dr. Roland R. Read who prepared the other derivatives of 1,3-bis-(2-ethylhexyl)-5-amino-5-methylhexahydropyrimidine reported in this paper.

SUMMARY

A series of variously substituted hexahydropyrimidines was tested for in vitro antimicrobial activity. One of the most active in vitro antimicrobial compounds of this series was 1,3-bis(2-ethylhexyl)-5-amino-5-methylhexahydropyrimidine (W1435). In the 1,3-bis- positions, the most active substitutents were the octyl radicals (as the 1-methylheptyl and the 2-ethylhexyl). When the 1,3-bis- substitutions were in the active range the 5-hydrogen, 5-methyl, and 5-ethyl substituents produced derivatives, all of which proved to be active. The 5-amino derivative was somewhat more active than the 5-nitro derivative. Salt formation with selected organic acids often produced compounds exhibiting enhanced antimicrobial activity. Some chelating agents enhanced the in vitro activity of the derivatives tested; outstanding in this regard was diethylenetriaminepentacetic acid. Sensitive organisms tested did not develop resistance readily to the 1,3,5-substituted hexahydropyrimidine compounds. The low incidence of development of resistance by sensitive organisms to 1,3-bis-(2-ethylhexyl)-5-amino-5-methylhexahydropyrimidine and its trihydrochloride derivative has been confirmed by many investigators.

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DISCUSSION

DR. KATZ: I'd like to ask Dr. Barkley if he has any in vivo studies with the hexetidines.

DR. BARKLEY: I don't have the results of those studies here. There have been quite a number made and reported in separate papers, however.

DR. KATZ: The reason I asked the question is that we were enchanted by the in vitro studies of the hexetidines and tried the use of some of them in infections that appeared to be susceptible in vitro. The conclusion was that these drugs were very good for treating test-tubes but not for treating patients. I don't know whether any method has been developed for measuring blood levels but at the time we did the study none was available.

DR. BARKLEY: Since I haven't done the in vivo work, I can't give all the data on it. The lipoproteins seem to reduce the activities greatly but oral efficacy would be questionable.

DR. SCHWARTZ: We spent a number of years studying these compounds and many derivatives of them. It seems that they are not effective orally as chemotherapeutic agents in treatment of infectious diseases. It seems that these materials are very actively bound by lipoproteins and they seem to be bound by lipoprotein in an irreversable manner, which doesn't permit absorption into the gastrointestinal tract. Now we also know that these materials may interfere with bacterial action by interfering with protein synthesis. This has been demonstrated many times by the work of Halvorson and his group and many others. They are not recommended and they are not being used as systemic agents but are being used very effectively as topical agents.

GENE-CONTROLLED RESISTANCE TO POISONS IN SACCHAROMYCES

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INTRODUCTION

A poison is an agent which produces an injurious effect by chemical action. The different individuals of a species or even of a genus are generally assumed to be sensitive to about the same concentration of a given toxic agent when treated under uniform conditions. It is on this supposition that the lethal dose is calculated and that lists in reference books specify the concentration of different poisons tolerated by Saccharomyces cerevisiae. Differences among members of the same species are generally inferred to be due to some environmental effect or some past experience such as immunization rather than to a hereditary difference. The study of sulfanilamide- and penicillin-resistant microorganisms raised the possibility that individuals or clones of a single species might differ markedly with regard to sensitivity and resistance to a specific poison. We have recently begun to study the hereditary differences between different sibling yeast cultures with regard to sensitivity or resistance to a variety of different poisonous agents. Wide differences with regard to sensitivity to a given poison were found among sibling cultures of our breeding stock of Saccharomyces. This breeding stock of Saccharomyces, in which several thousand sibling cultures were tested for tolerance to various toxic agents, was obtained by interbreeding six or seven different "species" of Saccharomyces. Gene-controlled differences in sensitivity to copper, cadmium, actidione, caffeine, and nicotine among siblings of this stock have been reported [1-5].

A culture was designated resistant if it continued to grow in the presence of a given concentration of the reagent; a sensitive culture was one which was incapable of continued growth in the presence of the reagent at the same concentration. This procedure requires a uniform inoculum and a uniform nutrient medium since a large inoculum or a favorable medium would require higher concentrations of the toxic agent to produce a lethal effect. A large inoculum might detoxify much of the toxic agent and permit growth. It was possible to distinguish sensitive from resistant by testing each culture over a wide range of concentrations and finding concentrations at which resistant cultures grew and sensitive failed to grow. This made it possible to detect Mendelian segregation by testing all the members of a family at the critical concentration [6].

In some pedigrees the cultures fell into the two categories of resistant or sensitive to certain poisons but simple Mendelian control of these differences could not be demonstrated. It is possible that these were instances in which more than one gene was involved or that resistance had a nongenic basis.

MATERIALS AND METHODS

Modified Burkholder's medium and nutrient agar were used. The ingredients are listed by Lindegren [7]. Sensitivity was tested by three methods.

Agar-plate method (A.P.). A specific concentration of the poison was incorporated into nutrient agar and cell suspensions were streaked on the agar. The inoculated plates were incubated at 30 C. Resistant cultures grew confluently while sensitive cultures failed to grow or showed very scant growth after 24 to 48 hr of incubation.

Paper-disc method (P.D.). Cell suspensions were diluted in sterilized water to a Klett reading of 200 ± 20. An inoculum of 0.2 ml was spread on a nutrient agar plate and the surface was dried by allowing the plate to stand at room temperature for an hour. Sterilized paper discs were saturated in a solution of the poison and placed on the previously seeded agar surface. The plates were read after 48 to 72 hr incubation. Resistant cultures grew up to the edge of the disc while a clear zone around the disc indicated sensitivity to the toxic agent.

Test-tube method (T.T.). Cell suspensions were diluted in sterilized water to a concentration such that an inoculum of 0.05 ml contained approximately $3 \cdot 10^5$ cells. Tubes of complete natural medium, free from the poison, served as controls. Both the control tubes and the tubes containing poison were inoculated with 0.05 ml cell suspension and incubated at 30 C. The tubes were compared with the Klett-Summerson photoelectric colorimeter after 24, 48, 72, and 96 hr of incubation. The turbidity reading was used as an index of growth. Cultures which were not able to grow or which grew very slightly in the medium containing toxic agent in 72 to 96 hr were scored sensitive.

Both the agar-plate and paper-disc methods have the advantage that resistant secondary colonies originating by mutation or adaptation are easily detectable. The paper-disc method is especially preferred when poisons are insoluble or form precipitates or color in the medium but the concentration of the toxic agent can be more precisely controlled and the amount of growth can be determined numerically by the test-tube method.

Since the toxic effect of a poison is measured by growth inhibition, many factors that will enhance or depress the growth should be avoided. A control without the poison is run at the same time and the same inoculum is used in both the control and the tube containing the toxic agent.

Various concentrations of the poison are tested to find one which inhibits sensitive cells but which permits resistant cells to grow. In many instances the intermediate range between the ineffective concentration and lethal concentration for both sensitive and resistant culture is wide. However, in some instances the nontoxic and the lethal dose are very close.

More than 70 different poisons were tested on many hundreds of different cultures. These included alkaloids, antibiotics, metals, alcohol, halogens, and various organic compounds. The resistant cultures grew at the critical concentration within 24 hr and most of the toxic agents suppressed the growth of sensitive cultures for at least 3 or 4 days. Mutation to resistance or adaptation to the poison may occur after 4 days in many instances. Hence, the time at which readings are made may be important. For example, clearcut segregation for ethyleneimine can be obtained only within 24 to 48 hours because mutation or adaptation occurs frequently.

EXPERIMENTAL RESULTS

Ten gene-controlled poisons. Table 1 shows the critical concentrations of ten toxic agents which distinguish sensitive from resistant cultures in the breeding stock and the different methods by which the analyses were made. The first six substances listed have been reported previously and the findings concerning them confirmed and extended. It is particularly interesting that resistance to such closely related ions as cobalt and copper should be controlled by separate, different genes. This suggests that two different critical enzymes produced by two different genes are each more specifically sensitive to Cu or Co than any of the other multitudinous enzymes present in the cell and that the defective cell produces the enzyme in significantly lower concentrations than the normal cell, with the result that the defective culture can be discriminated from the normal culture only by the presence of the particular ion. Since the possible number of different poisons is so great, especially when one considers the large number of organic compounds which are available, this procedure for discriminating different genotypes may turn out to be very useful.

Table 1. The Differentiating Concentration of Ten Toxic Agents at Which Sensitive and Resistant Cultures of Saccharomyces Can be Distinguished. The Differences Are Each Controlled by Single Different Genes

Toxic agents	Concentration, ppm	Method
Alkaloids		
Caffeine	30,000	T.T.
Nicotine	50,000	P.D.
Antibiotic		
Actidione	1	T.T.
Metals		
Cadmium nitrate	200	T.T.
Cobalt chloride	10,000-25,000	P.D.
Copper sulfate	127	A.P.
Organic compounds		
Allyl glycine	400-450	T.T.
3-Amino triazole	15,000	T.T.
Benzimidazole	750	T.T.
Ethyleneimine	400-450	T.T.

The four organic compounds, allyl glycine, 3-amino triazole, benzimidazole, and ethyleneimine, were all tested in extensive pedigrees which showed them to be controlled by single-gene differences, each of which was different from the other. All of the ten agents listed are controlled by separate, different genes. Since these genes can be detected only by treatment with the poison, it was assumed that in each case the recessive produces an amount of enzyme sufficient for growth in the absence of the poison but that the defective cell is inactivated when the poison is present. In addition to these ten, regular segregation was obtained by testing with appropriate concentrations of isopropanol and sodium vanadate.

Toxic agents not demonstrated to be under single-gene control. Ten toxic agents were tested and found to divide yeasts into two classes, sensitive and resistant. Matings of sensitive with sensitive and resistant with resistant produced offspring like the parents, indicating clearly that the differences were hereditary (Table 2). However, segregations were so irregular that control of a single gene could not be discriminated. These agents may open up the possibility of extending the study of the cytoplasmic components of the yeast cell.

Juglone and menadione. These compounds, which are related to each other, are interesting since they act on yeasts in exceedingly low concentrations. The

Table 2. Susceptibility of Saccharomyces. Sensitivity and Resistance Not Under the Control of a Single Gene

Toxic agent	Differentiating concentration, ppm	Method
Canavanine	100	T.T.
Dimethyl sulfate	2,523	T.T.
Endomycin	75	P.D.
8-Hydroxyquinoline	5	T.T.
Nickel nitrate	40,000	P.D.
Paranitrophenol	50	T.T.
Propamadine isethionate	1,750	P.D.
Rimocidin	9	P.D.
Salicylanilide	20	T.T.
Undecylenic acid	10	T.T.

range in concentration is dependent upon whether a synthetic or a natural substrate is used; the yeast is killed more readily in a synthetic than in a natural medium. No data have been collected yet concerning the gene-controlled aspects of juglone or menadione toxicity. The facts suggest that these drugs may prove useful for the treatment of yeast or fungal diseases.

Substances not toxic to yeast. Many toxic agents were tested and did not inhibit the growth of different yeast cultures at the test concentrations (Table 3).

Toxic agents which produce respiratory deficiency in yeast. One of the commonest hereditary changes induced in yeast by the treatment with poisons is respiratory

Table 3. Substances Not Toxic to Yeasts

Substance	Greatest concentration tested, ppm
Quinine sulfate	500
Albamycin	50,000
Cathomycin	50,000
Streptogramin	100,000
Strontium	1,000
Magnesium sulfate	2,000
Barium chloride	1,600
Chlorohydrate	1,000
Sodium oxalate	500
Sodium pirate	100
Sulfanilic acid	100
Hexamethylenetetramine	500
Tyramine	300
d-β-Phenyl lactate	300
Primoquine diphosphate	200
Stylomycin	200
Usnic acid	1,000
Aminopterin	Undiluted
Ginger oil	Undiluted
4-Aminotriazole	15,000
Azaguanine	150
Azathymine	1,000

deficiency. Stier and Castor [8] induced respiratory deficiency with cyanide and Whelton and Phaff [9] showed that ethylene oxide is extremely efficient in producing the same effect. Ephrussi [10] found that respiratory deficiency is induced by a variety of flavins. It is also induced by manganese, cobalt, copper, and nickel ions [11]. Lindegren and Hino [12] showed that anaerobiosis caused respiratory deficiency and Lindegren, Shult, and Desborough [13] described the induction of respiratory deficiency by adaptational stress under the toxic action of caffeine. Propamidine isethionate is of special interest for it induces respiratory deficiency in practically all cells at a concentration of 1750 ppm. This phenomenon is of interest because of its relation to Warburg's theory of the origin of cancer.

DISCUSSION

Poisons may assume a leading role in genetical analysis. The work of Beadle and Tatum established the "one-gene, one-enzyme" theory and this concept made it possible to discriminate organisms which were either capable or incapable of synthesizing a variety of nutrilites. The discriminations were sharp and clear-cut. The synthesizing organism grew well in medium lacking the nutrilite while the nonsynthesizer failed to grow in the same medium. The number of nutrilites for which a culture can be characterized is limited since less than 20 amino acids and a relatively smaller number of B-vitamins and other nutrilites are available while the number of poisons is practically unlimited. The evidence that sensitivity to many toxic agents is gene controlled suggests that the toxic agent inactivates the enzyme produced by the gene. The sensitive cultures grow well in the absence of the poisonous agent and this suggests that the difference between the sensitive and the resistant culture is not one of absence and presence of an enzyme but a difference in the amount of enzyme present. It may be inferred that the sensitive culture produces an amount of enzyme which can be inhibited by a specific concentration of poison while the resistant form produces a larger amount or a more stable enzyme which is not similarly inhibited. The relatively high frequency of gene-controlled sensitivity to poisons suggests that the frequency of instances in which normal and defective genes differ in this quantitative manner may be very high and that eventually this may become one of the most fruitful techniques of genetical analysis. It should be pointed out, however, that the diagnosis depends upon the amount of inoculum, the concentration of the toxic agent, and the time when the sensitive and resistant cultures are compared and that these details must be worked out carefully before the diagnosis can be made.

ACKNOWLEDGMENT

These data have been collected over several years from the work of Mrs. Lindegren and her assistants. Mr. Yu. Lin Hwang was very helpful in organizing the data. The author wishes to acknowledge the helpful discussions with Mr. Robert Drysdale.

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DISCUSSION

DR. EMMONS: This is very interesting and precise work, Dr. Lindegren. We have been familiar with work coming from your laboratory for many years. Your paper is of interest, even though it has no immediate medical significance.

IN VITRO COMPARATIVE STUDIES OF THE ANTIFUNGAL ACTIVITY OF TRICHOMYCIN, PIMARICIN, AND GRISEOFULVIN

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INTRODUCTION

Trichomycin is an antibiotic obtained from cultures of Streptomyces hachijoensis. It was isolated for the first time in 1952 by Hosoya et al. [1] who discovered the microorganism in a soil sample from a Japanese island. Its chemical structure is not yet completely known but according to Hattori et al. [2] the molecule is a conjugated heptaene. As a powder it is stable but in solution it progressively loses activity. In vitro and in vivo experiments demonstrated that trichomycin is highly effective against Candida albicans [3, 4] and other yeasts and against Trichomonas vaginalis [5]. It is also active against some pathogenic fungi [7, 8] and several protozoan parasites, especially flagellata and amoeba [6].

Pimaricin, another new antibiotic, was isolated in 1955 at Delft (Netherlands) by Strucky and Waisvisz [9] from a culture of Streptomyces natalensis obtained from a soil sample of South Africa. Chemically, pimaricin is a crystalline, colorless substance, its complex molecule ($C_{34}H_{49}NO_{14}$) containing a system of four conjugated double bonds (tetraene). Biological investigation shows that pimaricin exhibits a broad-spectrum of antifungal action [9]. It is effective in inhibiting the growth of many yeasts and saprophytic as well as pathogenic fungi which produce plant diseases and skin diseases in man and animals. It is also active against T. vaginalis and other flagellata [10].

Griseofulvin is the well-known antibiotic obtained from cultures of Penicillium griseofulvum, which was discovered by Oxford et al. [11] in 1939 and whose antifungal properties were studied in vitro by Brian et al. [12] in 1946. However, it became an important therapeutic agent only after the investigations carried out by Gentes [13] in 1959. Griseofulvin is effective against dermatophytes, mainly Trichophyton and Microsporum [14].

The present study was undertaken to compare the in vitro activity of these three compounds and their relative antifungal selectivity.

MATERIALS AND METHODS

Microorganisms. C. albicans Yu-1200, which is the standard used to determine the activity of trichomycin, was obtained from Fujisawa Pharmaceutical Co., Osaka, Japan. The other organisms were obtained from L.I.F.E.'s stock culture collection.

Agents. Solutions of the antibiotics studied were prepared by adding trichomycin to a diluent consisting of a 1 : 1 mixture of acetone and water with 8 mg% of NaOH. A concentration limit of 2.7 mg/ml (when 1 μg = 3.7 U) was obtained from which the necessary dilutions were made. Pimaricin was dissolved in pure glycerol to achieve a concentration of 10 mg/ml. Griseofulvin was dissolved in pure methyl alcohol to a concentration of 2 mg/ml. Fresh solutions were used.

Technique. After preliminary assays the following standard technique was adopted. Cultures were made in mycophyl broth (Baltimore Biological Laboratory). Forty-nine milliliters of broth were distributed into glass flasks, each containing 1 ml of the antibiotic solution. For Candida strains 1 ml of a 1 : 1000 dilution of a 24-hr tryptose-phosphate broth culture was used as the inoculum. For the other fungi the inoculum was a standard 3-mm plug from a 25- to 30-day-old culture of the organism on Sabouraud's maltose agar (Difco). Cultures were incubated at 28 C and the growth was recorded at 48-hr intervals. However, only the results recorded on the sixth day were used for statistical analysis. Five duplicates for each strain and each antibiotic concentration were used. For each series of concentrations there was a control culture which received the respective diluent but not the antibiotics. At least four antibiotic concentrations were tried.

The 100% minimal inhibitory concentration (MC_{100}) was found for each organism as the lowest concentration of the antibiotic at which there was no visible growth of fungi or turbidity in the case of Candida. A 10% minimal inhibitory concentration (MC_{10}) was also found as the lowest concentration which allowed approximately 90% growth, measured according to the diameter of the colony in the case of fungi and turbidimetrically in the case of Candida. Relative potencies as well as the ratio MC_{100}/MC_{10} were calculated.

RESULTS

Figure 1 and Table 1 present the concentrations required to obtain the desired two levels of growth inhibition, namely, 10 and 100% inhibition. Within the limits of the concentrations studied all organisms but one were completely inhibited in their growth. Candida was only slightly inhibited by griseofulvin despite the high concentrations used.

Figures corresponding to the ratio MC_{100}/MC_{10} showed that the more selective a compound was in inhibiting the growth of an organism, the lower was its numerical value. For instance, trichomycin presented a ratio of 3 to 5 when used upon Candida and reached a value of 36 when used upon *Trichophyton violaceum*. In other words, when an antibiotic is not very selective, it is capable of producing a slight inhibition of growth at a relatively low concentration but 100% inhibition is obtained only at a very high concentration.

There is no agreement as to classification of organisms according to their susceptibility to the three antibiotics studied. Arbitrarily we have considered as

Table 1. Antifungal Activity of Trichomycin, Pimaricin, and Griseofulvin

Organism	Strain	100% Minimal inhibitory concentrations (MC_{100}), μg/ml and ratio MC_{100}/MC_{10}					
		Trichomycin		Pimaricin		Griseofulvin	
		MC_{100}	Ratio	MC_{100}	Ratio	MC_{100}	Ratio
C. albicans	Yu-1200	0.35	3.5	2.0	4.0	>1000	–
C. albicans	A-101	2.1	5.1	3.0	3.0	>1000	–
M. canis	B-1	150.0	30.0	25.0	6.3	10	10
M. canis	G-2	200.0	28.0	50.0	10.0	25	10
T. violaceum	Z-17	90.0	36.0	10.0	10.0	50	12
T. schoenleini	A-1	160.0	26.0	50.0	10.0	25	10
T. mentagrophytes	AHC-1	250.0	25.0	50.0	14.0	25	10
A. fumigatus	9194	250.0	10.0	25.0	10.0	150	30

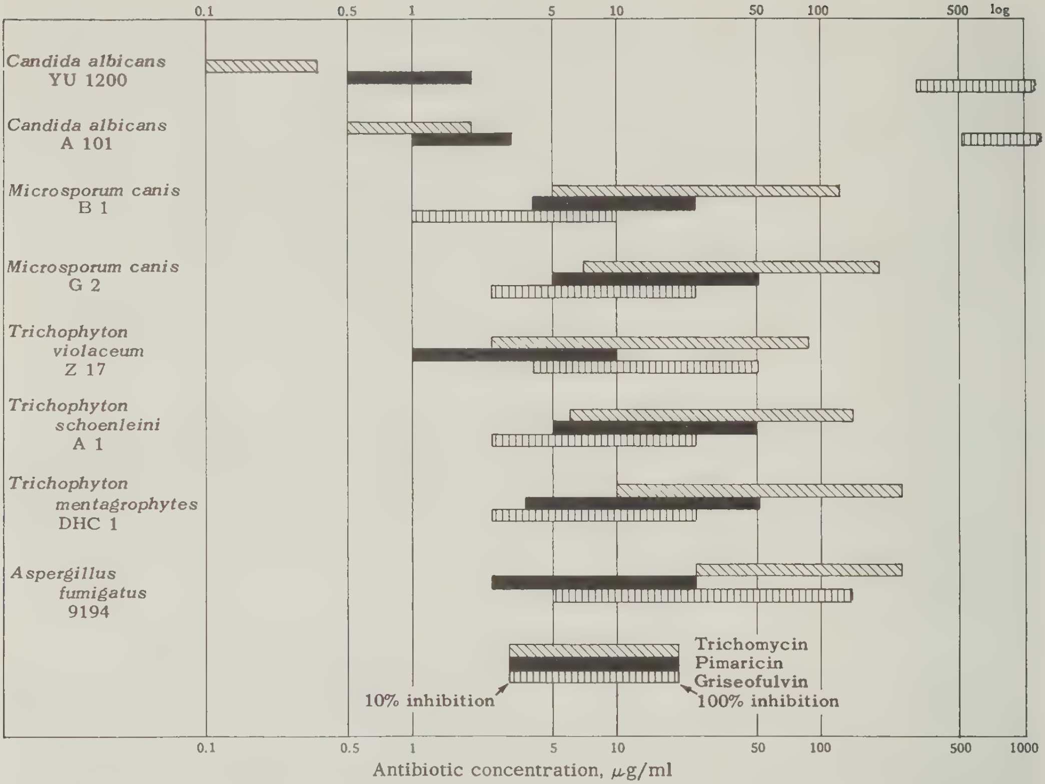

Fig. 1. Minimal concentration required to produce 10% (at left side) and 100% (at right side) inhibition of growth.

Table 2. Degree of Susceptibility of the Studied Microorganisms to Trichomycin, Pimaricin, and Griseofulvin

Drug	Organisms			
	Highly susceptible, <1 to 9.9 μg/ml	Very susceptible, 10 to 99 μg/ml	Moderately susceptible, 100 to 499 μg/ml	Resistant, >500 μg/ml
Trichomycin	C. albicans	T. violaceum	M. canis T. schoenleini T. mentagrophytes A. fumigatus	
Pimaricin	C. albicans	T. violaceum M. canis A. fumigatus T. schoenleini T. mentagrophytes		
Griseofulvin		M. canis T. schoenleini T. mentagrophytes T. violaceum	A. fumigatus	C. albicans

"highly" susceptible those organisms for which the MC_{100} is less than 10 μg/ml; "very" and "moderately" susceptible and "resistant" those for which the MC_{100} was between 10 and 99 μg/ml, 100 to 499 μg/ml, and more than 500 μg/ml, respectively (Table 2).

Table 3 shows the relative activity of the three compounds on each organism. Trichomycin was the most active agent in inhibiting the growth of *C. albicans*. There were quantitative differences according to the strain of Candida but the potency of trichomycin was 2 to 5 times higher than that of pimaricin. Griseofulvin was almost inactive against this microorganism.

Table 3. Relative Antifungal Activity of Trichomycin, Pimaricin, and Griseofulvin

Organism	Antibiotics* (in sequence of their relative activity)	Relative activity
C. albicans Yu-1200	T > P > G	28.5 : 5.0 : 0.01
C. albicans A-101	T > P > G	4.7 : 3.3 : 0.01
M. canis B-1	G > P > T	15.0 : 2.5 : 1
M. canis G-2	G > P > T	8.0 : 2.0 : 1
T. violaceum Z-1	P > G > T	9.0 : 5.0 : 1
T. schoenleini A-1	G > P > T	6.4 : 2.0 : 1
T. mentagrophytes AHC-1	G > P > T	10.0 : 2.0 : 1
A. fumigatus 9194	P > G > T	10.0 : 6.0 : 1

* T, trichomycin; P, pimaricin; G, griseofulvin.

Griseofulvin was the most active agent against *Microsporum canis*, its potency being 4 to 6 times higher than that of pimaricin and 8 to 15 times higher than that of trichomycin.

Pimaricin was the most active against *T. violaceum*, being twice as potent as griseofulvin and 9 times more potent than trichomycin.

Griseofulvin was the most active agent and trichomycin the least against *Trichophyton schoenleini* and *Trichophyton mentagrophytes*.

On *Aspergillus fumigatus*, pimaricin was the most active agent, griseofulvin was second in activity, and trichomycin last, being only one tenth as potent as pimaricin.

DISCUSSION

Among the yeasts and fungi tested only *C. albicans* was "highly" susceptible to both trichomycin and pimaricin. Lutz and Witz [15] reported that trichomycin was some 30 to 70 times more active against *C. albicans* than its congener, nystatin, which was considered, until the discovery of trichomycin, the most selective antibiotic for inhibiting such an organism. At the present time trichomycin is the most potent agent against Candida, a fact that has been confirmed consistently in the therapeutic field [4, 5, 15].

None of the other pathogenic fungi appeared as "highly" susceptible to any of the three antibiotics described. If, for example, the minimal concentration of penicillin required to kill susceptible bacteria, is compared with the concentration of griseofulvin or pimaricin required to completely inhibit the growth of Trichophyton or Microsporum, it is easy to see that these latter antibiotics are not extremely potent agents.

Certainly the introduction in therapy of these new antibiotics represents important progress in the treatment of cutaneous fungus diseases. However, we have still to search for more potent and more selective agents.

SUMMARY

Antifungal activity of trichomycin, pimaricin, and griseofulvin was studied by using the in vitro method of serial dilutions. The 10 and 100% minimal inhibitory concentrations were found for each antibiotic and each organism tested.

Trichomycin was the most potent agent against *Candida albicans*. Pimaricin, according to the strain of Candida, was 2 to 5 times less active than trichomycin and griseofulvin was almost inactive.

Griseofulvin was the most active agent against *Microsporum canis*. Its potency was 4 to 6 times higher than that of pimaricin and 8 to 15 times higher than that of trichomycin.

For *Trichophyton violaceum*, pimaricin was twice as potent as griseofulvin and trichomycin was the least active agent. On *Trichophyton schoenleini* and *Trichophyton mentagrophytes*, griseofulvin was the most active agent and trichomycin the least.

Pimaricin was the most active agent and trichomycin the least against *Aspergillus fumigatus*.

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DISCUSSION

DR. KATZ: Dr. Muggia, can you tell us whether these drugs have had clinical trials, particularly trichomycin, and either clinical trial or in vivo use in experimental infections in mice?

DR. MUGGIA: I was not there myself at the time of the studies and I am not aware that they have tried it clinically.

DR. EMMONS: We are indebted to Dr. Muggia for presenting this paper and he has an adequate explanation for his inability to answer all questions but if there are any questions or any comments from anyone in the audience we would be glad to hear them. The role of griseofulvin in the treatment of superficial fungus infections is certainly well established. We tried griseofulvin against the systemic mycoses as soon as it became available to us and found, as others have, that it was not effective in treatment of systemic mycoses. Trichomycin is available for experimental use now but we do not have conclusive results to report at this time.

DR. LYNCH: (Kansas City) We have had some experience testing this drug both in vivo and in vitro and at the present time I thought it might be interesting to point out some of our in vitro results. With our standard organism, Candida, that we use to compare all drugs, we found that a level of about 0.25 μg gives the same effect that would be given, let's say, by a weaker concentration of amphotericin B, so that in our hands it is not necessarily the most potent drug against that organism. There is an organism which is supplied by the company for testing this drug. It is a Japanese isolate of Candida and I'm not sure quite where they got it from but it is exceedingly sensitive to this drug, except that we have had trouble propagating the organism and comparing it to others. I was just wondering whether you might know whether that was the particular isolate that was used in these studies.

DR. MUGGIA: I'm sorry. Once again, I can't answer that question.

PERIMYCIN, A NOVEL TYPE OF HEPTAENE ANTIFUNGAL ANTIBIOTIC

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INTRODUCTION

Perimycin, an antifungal antibiotic, was isolated in crude form by Wooldridge and Hoffman in 1952. These investigators produced this antibiotic from an unidentified Streptomyces found as a contaminant on a culture plate of a skin scraping. The organism was subsequently identified by J. W. Foster of the University of Texas as a hitherto undescribed species for which he suggested the name Streptomyces aminophilus. This species has been studied more recently in greater detail by Waksman and Kutzner and designated by them as Streptomyces coelicolor var. aminophilus. A detailed description of the organism is in preparation for publication.

The crude antibiotic, designated as antibiotic 1968, was reported by Oswald et al. [1] in 1955 as having promising in vitro antifungal activity and being akin to ascosin. Campbell et al. [2], at the same time, showed that antibiotic 1968, crude as it is, markedly reduced mortality of mice with experimental histoplasmosis, sporotrichosis, and moniliasis.

The program of the 1960 Conference on Antimicrobial Agents (Washington, October 26, 1960) refers to this antibiotic as aminomycin. This name has been withdrawn since it was found that Brockmann and Schmidt-Kastner [3] used this name for the antibiotic, valinomycin.

In the present paper the authors report on the purification of crude perimycin and demonstrate that the antibiotic in pure form may be differentiated from other known heptaenic antifungal antibiotics by its physical, chemical, and biological properties.

EXPERIMENTAL

Purification of perimycin. In one experiment a 10-g sample of crude perimycin (Warner-Lambert lot No. WXa2412), estimated to be of 15 to 20% purity, was stirred with 50 ml of methanol and 10 ml of ethyl acetate. The mixture was boiled for 5 min on a steam bath and filtered. The filtrate was cooled in a dry ice-acetone bath and the resulting precipitate was centrifuged at −20 C and washed repeatedly with ethyl ether. The wet precipitate was shaken with 990 ml of a 1 : 1 mixture of 1-butanol and water until it dissolved. The resultant solvent mixture was adjusted to pH 10−11 with 1% sodium hydroxide and shaken again for 5 min. The butanol layer was separated, washed with water until neutral, and concentrated under reduced pressure at room temperature to a final volume of 80 ml. During concentration, partial precipitation of perimycin resulted. This was completed by the addition of 300 ml of ethyl ether. The precipitate was centrifuged, washed with ethyl ether, and dried under vacuum to yield 1.85 g of a yellow amorphous solid of 71% purity. Concentration of the mother liquor with subsequent precipitation by addition of an excess of ethyl ether produced an additional 0.15 g of perimycin of 71% purity.

To achieve further purification countercurrent-distribution studies with the solvent system, pyridine—ethyl acetate—water (3.5 : 6.5 : 8.3), were made. In one experiment with perimycin of 71% purity 200 transfers were carried out in a 200-tube 40-ml upper- and lower-phase apparatus. Perimycin exhibited a peak concentration at tube No. 137, corresponding to a calculated partition coefficient of 2.17. The contents of tubes 120 to 150 were combined, extracted with petroleum ether to remove pyridine and ethyl acetate, and the aqueous phase lyophilized. A concentrated solution of the dried perimycin residue was made in boiling methanol and a precipitate was obtained on cooling to approximately −10 C. After centrifugation the precipitate was washed with ethyl ether and dried under vacuum. The resultant perimycin was an amorphous golden-yellow solid exhibiting an $E_{1cm}^{1\%}$ of 1000 at 383 mμ in methanol, which remained constant upon further reprecipitations from methanol. Additional countercurrent-distribution studies with the solvent system, chloroform—methanol—borate buffer (2 : 2 : 1), pH 8.2, confirmed the existence of one heptaenic component. Based on the countercurrent distribution and ultraviolet spectrophotometric data this preparation of perimycin was considered to be pure.

Isolation of p-aminophenylacetone. Two grams of pure perimycin was heated at 100 C for 15 min with 400 ml of 10% sodium hydroxide and the resultant mixture was centrifuged. The supernate was extracted twice with 100-ml aliquots of chloroform. The combined extracts were washed with 50 ml of water and evaporated to dryness under reduced pressure. The resultant residue was dissolved in hot water and insoluble oily impurities were removed by centrifugation while still warm. The supernatant liquid was evaporated in vacuo, and the residue was crystallized from water to give colorless crystals melting at 101–102 C.

The microanalytical data are as follows: Found; C, 72.35; H, 7.63; N, 9.00; M.W. (Rast), 154. Calculated for $C_9H_{12}ON$; C, 72.00; H, 8.00; N, 9.30; M.W. (Rast), 150. Maximum ultraviolet light absorption at 330 mμ. $E_{1cm}^{1\%}$ = 1740 (methanol).

N-acetyl p-aminobenzoic acid was obtained by chromic acid oxidation in glacial acetic acid of the N-acetyl derivative of this product. This identification was based on a comparison with synthetic N-acetyl p-aminobenzoic acid by paper chromatography employing the solvent system tetrahydrofuran—3N ammonium hydroxide (4 : 1), by ultraviolet absorption spectroscopy at the maximum of 267 mμ, and by mixed melting-point determinations (mp 250 C, no depression).

The oxygen in the product, $C_9H_{12}ON$, was shown to be present as a carbonyl group by the formation of a dinitrophenylhydrazone. The above evidence and the formation of iodoform by treatment of the product with iodine and alkali proves that the product is p-aminophenylacetone (Fig. 1).

Amino sugar isolation. Pure perimycin (500 mg) was hydrolyzed with 25 ml of 2N sulfuric acid in 50% aqueous methanol for 10 min at 70 C. Dark-colored degra-

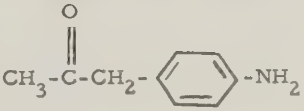

Fig. 1. Para-aminophenylacetone.

dation products were removed by centrifugation; the supernatant solution, after dilution by two volumes of water, was extracted with 1-butanol, which was discarded. The aqueous phase was neutralized with barium carbonate, concentrated under vacuum, and finally freeze-dried. An amorphous product was obtained by applying the dried hydrolysate directly to a cellulose-powder chromatographic column and developing the column with the solvent system, 1-butanol—pyridine—water—acetic acid (6 : 4 : 3 : 1). This product had the properties of an amino sugar and a crystalline N-carbobenzoxy derivative was prepared by treating the pure amino sugar with carbobenzoxy chloride at pH 8.0—8.5 and extracting into ethyl acetate. The amino sugar is not a 6-desoxy sugar since acetaldehyde could not be obtained from the sugar by treatment with sodium periodate. It can also be differentiated from mycosamine by paper chromatography using the solvent system previously used for the cellulose-powder-column chromatography of the amino sugar.

Differentiation of perimycin by countercurrent distribution. Countercurrent distribution studies using 200 transfers were made in two different solvent systems; A, pyridine—ethyl acetate—water (3.5 : 6.5 : 8.3), the system previously used for the purification of the antibiotic; and B, chloroform—methanol—borate buffer (2 : 2 : 1), pH 8.2, the system previously used for the proof of homogeneity of pure perimycin. Figure 2 represents the distribution of candidin, candicidin, and perimycin in solvent system A. The partition coefficients of these three heptaene antibiotics in the two solvent systems cited were as follows: System A; candidin, 0.48; candicidin, 1.25; perimycin, 2.2. System B; candidin, 4.4; candicidin, 0.9; perimycin, 0.1.

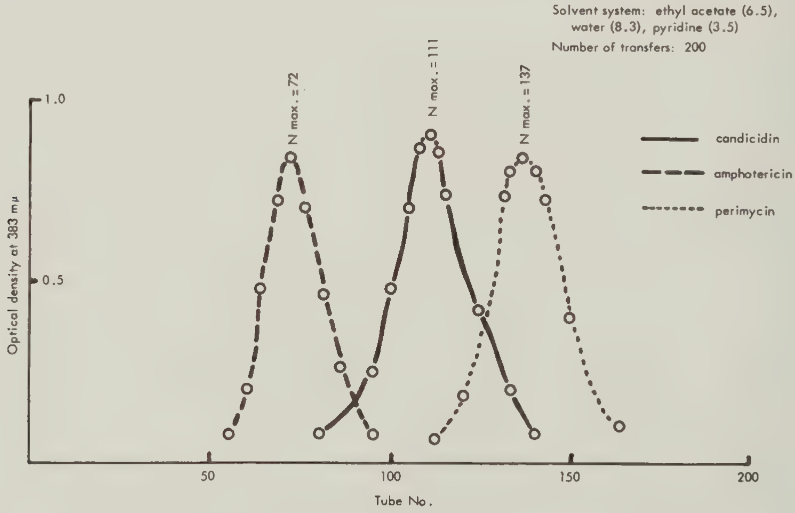

Fig. 2. Countercurrent distribution of three subgroups of heptaenes.

Differentiation of perimycin by paper chromatography. Perimycin was compared with other heptaene antibiotics by descending paper chromatography with the solvent system, pyridine—1-butanol—water (4 : 6 : 5). The position of the antibiotics was detected by means of bio-autography using Candida albicans 204. The following R_f values were obtained: candidin, 0.5; amphotericin B, 0.52; candicidin and trichomycin, 0.61; perimycin, 0.80. Figure 3 gives the paper chromatographic comparison of the various heptaene antibiotics.

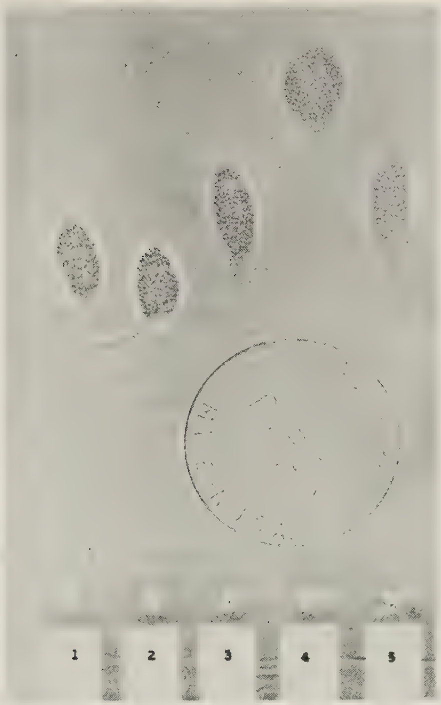

Fig. 3. Paper chromatography of heptaenes. Solvent system: pyridine (4); n-BuOH (6); H_2O (5). Bioassay with *C. albicans*: 1) Amphotericin B; 2) candidin; 3) candicidin; 4) perimycin; 5) trichomycin.

Biological methods. Stock solutions of pure perimycin (lot 5-4-60), pure candicidin (lot 1-9-59), pure candidin (lot 5-27-60), pure amphotericin B (lot 5-13-60), and 50% pure trichomycin (lot L-923R) were made in dimethylsulfoxide at the level of 1 mg/ml for all the antibiotics used, except for trichomycin, which was put in solution at the level of 2 mg/ml. Dilutions were made in the test medium to obtain the following final concentrations of the pure antibiotics: 1, 3,

Table 1. Antimicrobial Spectrum of Perimycin, Streak-Dilution Assay on Peptone-Meat Extract-Glucose Agar, Readings after 48 hr at 28 C

Organism	Total inhibitory concentration, μg/ml
Saccharomyces cerevisiae 216	0.06
Candida albicans 204	0.06
C. tropicalis 205	0.30
Hansenula anomala 317	0.01
Aspergillus niger 3	0.01
Aspergillus niger 8	0.01
Aspergillus niger 13	0.03
Penicillium notatum 41	0.01
Fusarium oxysporum cubense 59	0.30
Mucor rouxii 80	3.0
Cunninghamella sp. 89	Growth at 10
Rhizopus japonicus 207	Growth at 10
Four Gram-positive bacteria	Growth at 50
Four Gram-negative bacteria	Growth at 50
Two mycobacteria	Growth at 50
Nocardia asteroides	Growth at 50
Three streptomycetes	Growth at 50

Table 2. Effect of Five Heptaenes Against Four Fungi, Tested by Streak-Dilution Assay on Peptone-Meat Extract-Glucose Agar

Antibiotic	Minimum inhibitory concentration, μg/ml*			
	Candida albicans 204	Saccharomyces cerevisiae 216	Aspergillus niger 13	Mucor rouxii 80
Perimycin	0.10	0.07	0.06	3.8
Trichomycin	0.03	0.03	0.50	3.5
Candicidin	0.04	0.03	0.83	1.27
Candidin	0.60	0.50	0.38	0.60
Amphotericin B	0.50	0.50	0.09	0.40

*Average of six assays. Readings after 48 hr at 28 C.

6, and 10 μg/ml or similar values in a different range such as 0.01, 0.03, 0.06, and 0.1 μg/ml. Assays were made on peptone-meat extract-glucose agar [4]. Stocks of the organisms listed in Tables 1 and 2 were grown on yeast extract-glucose agar for the fungi and on nutrient agar for the bacteria. The bacterial cultures were incubated at 37 C for 18 hr and the fungal cultures at 28 C for 24 to 48 hr before being suspended in water for use as inocula in the assay plates. Incubation of the assay plates was at 28 C for 48 hr before minimum inhibitory concentrations were estimated.

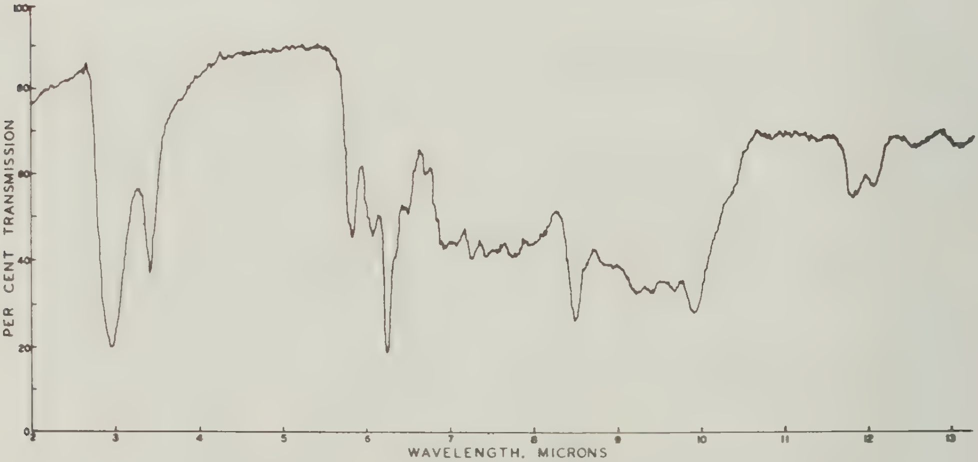

Fig. 4. Infrared spectrum of perimycin (KBr pellet).

DISCUSSION

Pure, though not crystalline, perimycin has the typical light absorption properties of heptaenes with major maxima at 361, 383, and 406 mμ ($E_{1\mathrm{cm}}^{1\%}$ at 383 mμ = 1000 in methanol). It does not have a definite melting point but decomposes slowly with darkening upon heating. It is insoluble in water, petroleum ether, ethyl acetate, and benzene. It is soluble in the following solvents in the presence of water: lower alcohols, pyridine, tetrahydrofuran, acetone, and dioxane. It is also soluble in methanol upon heating, in dimethylformamide, dimethylsulfoxide, and in the lower fatty acids. In general the solubility of perimycin in organic solvents is higher than that of other heptaenes. The infrared absorption spectrum, determined in potassium bromide pellets, shows sharp bands at 2.95, 3.43, 5.83, 6.05, 6.25, 6.50, 6.70, 7.25, 8.48, 9.90, 11.8, and 12.05 μ (Fig. 4). Analytical data: Found; C, 63.19; H, 8.28; O, 22.96; N, 3.38; C–Me, 5.67. Calculated for $C_{47}H_{75}O_{14}N_2$; C, 63.30; H, 8.40; O, 25.11; N, 3.14. The molecular weight was 948 by the perchlorate-titration method (calculated 892). The molecule contains four $C\text{-}CH_3$ groupings (Kuhn-Roth determinations).

Perimycin, unlike the other heptaenes, does not contain a carboxylic group. Like candicidin, for example, it contains two free primary amino groups. One of them is in the glycosidically linked amino sugar and the other in the p-aminophenylacetone moiety which is linked to the rest of the molecule through an aldol system.

In the light of what is known of the chemistry of heptaenic antifungal antibiotics, perimycin represents a novel type of structure. First, it does not contain mycosamine, which has been isolated from many nitrogen-containing polyenes such as nystatin, amphotericin B, candicidin, and candidin; it contains a different amino sugar, the structure of which has not yet been determined. This amino sugar has not been found heretofore in the molecules of other polyenes. Second, the aromatic moiety, p-aminophenylacetone, is different from p-aminoacetophenone which was isolated by Nakano et al. [5] from trichomycin and from candicidin. These structural differences result in paper-chromatographic (Fig. 3) and countercurrent-distribution behaviors (Fig. 2) of perimycin which are different from those of the other heptaenes.

As listed in Table 1, pure perimycin has the usual antimicrobial activity characteristic of heptaenic antifungal antibiotics. It is active against fungi but has no activity against bacteria and actinomycetes. The antifungal activity of pure perimycin was compared side by side with that of the other heptaenes available to us, namely trichomycin, candicidin, candidin, and amphotericin B. In this comparison two yeasts and two molds were used. Trichomycin and candicidin were the most active compounds against yeasts whereas candidin and amphotericin B were the least active compounds against these organisms (Table 2). Against the yeasts, the activity of perimycin was closer to that of trichomycin and candicidin than to that of candidin and amphotericin B. Just as amphotericin B can be differentiated from candidin by its higher activity against certain filamentous fungi [4] so perimycin can also be differentiated on the same basis from trichomycin and candicidin. In Table 2 the high activity of perimycin against Aspergillus niger No. 13 is noteworthy. In addition, perimycin was very active against two additional strains of A. niger (Table 1).

ACKNOWLEDGMENT

The authors wish to thank Mr. Vinayak V. Kane for the infrared analysis and Mrs. Eva M. Fekete for her technical cooperation. This investigation was supported in part by a research grant, No. E-2095, from the National Institute of Allergy and Infectious Diseases, Public Health Service.

SUMMARY

Perimycin, a heptaenic antifungal antibiotic produced by Streptomyces coelicolor var. aminophilus, has been obtained in pure form.

Perimycin is almost as active as trichomycin and candicidin against Saccharomyces cerevisiae and Candida albicans; it has about the same activity as

these antibiotics against *Mucor rouxii*. It is, however, more active than these antibiotics against *Aspergillus niger*. In the latter respect the activity of perimycin is similar to that of amphotericin B.

Perimycin is a basic heptaene, $C_{47}H_{75}O_{14}N_2$ (tentative), containing two primary amino groups, a number of C-methyl groups, and a number of hydroxyl groups. It does not contain a carboxyl group. The two amino groups are present in an aldolically linked p-aminophenylacetone and in a glycosidically linked amino sugar which differs from mycosamine. Perimycin is more soluble in organic solvents than the other heptaenes.

Perimycin has been differentiated from other heptaenes by paper chromatography, countercurrent distribution, and comparative chemical degradation.

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DISCUSSION

DR. KATZ: In the early days of No. 1968 (Nepera) we found it ineffective in systemic coccidiomycosis.

DR. SCHWARTZ: As you probably know, this antibiotic has a long, long history. The first crude material was tested in 1952 and somewhat purer materials were tested by Food and Drug from 1953 to about 1955. It was also tested in our laboratories for the last five years. The material has a long history and different activity depending upon which fraction and which preparation is prepared. We know it has a wide antifungal activity. We have not tested any material of the nature we speak about yet in the clinic.

DR. KATZ: I'm glad to have that clarification. We used it in 1957, but I didn't know what it was then.

DR. SCHWARTZ: I don't know where you got the material to test in the clinic in 1957 and this must be material which was made, I would say, in about 1955. The material we are speaking about today, and Dr. Borowski, I'm sure, has done a remarkable job in characterizing this material chemically, is a new antibiotic. It is quite different from the crude material which was presented in the data by Charlotte Campbell and her group in 1955 in terms of the efficacy of the crude material at that time against systemic fungal diseases.

THE EFFECT OF AMPHOTERICIN B UPON RENAL FUNCTION IN MAN

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INTRODUCTION

Amphotericin B is a polyene antibiotic which has been available since 1956 [1, 2]. It is synthesized by a species of Streptomyces and is the first drug that has been effective in the treatment of some of the deep mycoses in man [3, 4]. It is valuable in treatment of most cases of blastomycosis, cryptococcosis, histoplasmosis, and coccidioidomycosis. Several troublesome side effects have limited the clinical usefulness of this agent. Nausea, vomiting, chills, fever, and myalgia during administration occur in virtually every patient given the drug. A transient mild anemia occurs in a large proportion of patients [5] and elevation of the blood urea nitrogen (BUN) occurs frequently during treatment. This azotemia is almost always transitory, falling to normal a few days or weeks after treatment is stopped. No evidence of permanent renal damage from the drug has been presented. However, except for phenolsulfonphthalein (PSP) excretion and urea-clearance studies [4] the problem has remained largely unexplored. The present study was undertaken to evaluate the effects of amphotericin B upon the kidney, utilizing inulin and sodium para-aminohippurate (PAH) clearance determinations.

METHODS AND RESULTS

Fifteen patients† receiving amphotericin B for histoplasmosis, coccidioidomycosis, or cryptocococcosis have been studied. Measurements of the plasma clearance of inulin and PAH utilizing standard constant-infusion techniques, as outlined by Smith [6], have been carried out. Eight patients were studied while receiving treatment and seven patients were studied 47 to 428 days after the drug was stopped. The total dose of amphotericin B varied from 585 mg to 2425 mg, administered over a 30- to 90-day period. The results from all patients studied are summarized in Fig. 1. Normal inulin-clearance values after treatment were found in only two young men who were not seriously ill. These data indicate that renal plasma flow, as represented by PAH clearance, is affected to a lesser extent than is glomerular filtration rate and makes a smaller return toward normal after treatment is stopped. This suggests that decreased inulin and PAH clearances persist after treatment is stopped. Significantly decreased clearances have been observed in several patients as long as three months after termination of therapy.

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*The views expressed herein are the personal views of the Air Force author and are not to be construed as statement of official Air Force policy.

†Eight of these patients were studied through the courtesy of J. Lewis Yates, M.D., and the medical department of the Missouri State Tuberculosis Sanatorium, Mt. Vernon, Missouri.

Clearances done on a patient before, during, and after treatment are shown in Fig. 2. This patient was an apparently healthy young man who had a pulmonary coccidioidal cavity which was resected because of increase in cavity size and hemoptysis. Because of myalgia and bone pain he refused further therapy after only 653 mg of the drug. This patient was the only one observed who did not have an elevation of the BUN. After 16 days of treatment the inulin value had fallen to 75% of the pretreatment level and the PAH value to 74%. One hundred nineteen days after termination of treatment the inulin-clearance value had risen to above pretreatment levels while the value for PAH had returned to approximately the original value.

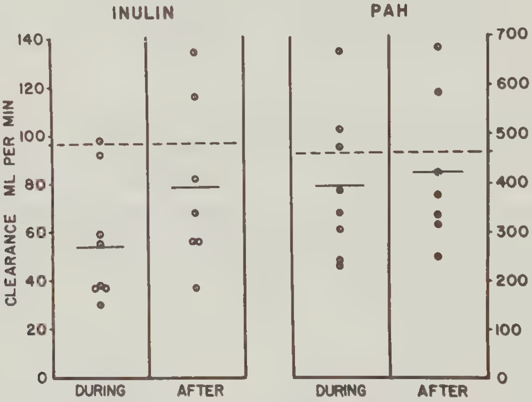

Fig. 1. Inulin clearance values during and after amphotericin B therapy are shown by the two bars on the left side. Corresponding values for PAH clearance are shown on the right. The dotted line on each bar is the lower limit of the normal range and the short solid line in each bar is the mean value for each group. Each open circle indicates the value for one determination on one patient.

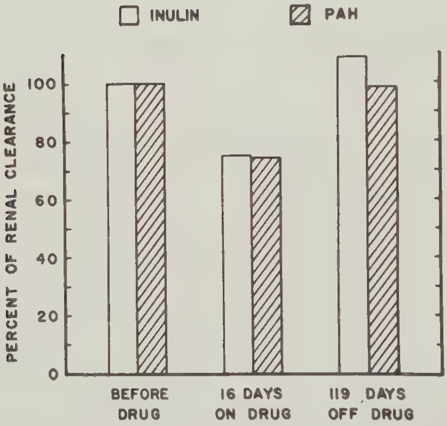

Fig. 2. Renal clearances of inulin (plain bar) and PAH (cross-hatched bar) before, during, and after amphotericin B therapy are shown. Initial values obtained before the drug was given are shown as 100% and subsequent values are represented as a percentage of the original value. The patient is a 27-year-old white male with active, cavitary pulmonary coccidioidomycosis.

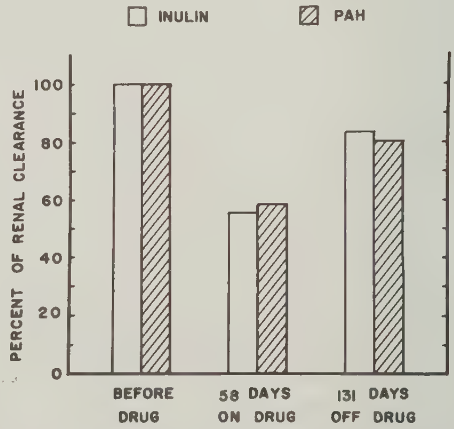

Fig. 3. Renal clearances of inulin (plain bar) and PAH (cross-hatched bar) before, during, and after amphotericin B therapy are shown. Initial values obtained before the drug was given are shown as 100% and subsequent values are represented as a percentage of the original value. The patient is a 55-year-old white female with disseminated coccidioidomycosis.

Figure 3 shows similar data for a middle-aged white female severely ill with disseminated coccidioidomycosis. After 58 days of therapy the inulin clearance was 55% of the value obtained before treatment while the PAH value was 58% of the pretreatment value. One hundred and thirty-one days after therapy was stopped the inulin clearance was 84% and PAH clearance was 80% of the pretreatment values.

DISCUSSION

These data indicate that amphotericin B exerts a pronounced effect upon the function of the kidney as measured by inulin and PAH clearances. In our patients inulin-clearance values were depressed to a proportionately greater degree than PAH clearance and showed a greater return toward normal values after therapy was stopped. Since inulin clearance measures glomerular-filtration rate and PAH clearance reflects renal plasma flow [6], these changes in renal function could be produced by diffuse vascular damage such as impairment of blood supply to the nephron.

Kidney biopsies were obtained by percutaneous needle puncture from three patients who were receiving amphotericin B therapy. These specimens showed subtle nonspecific degenerative changes in the proximal tubules, best seen with periodic acid Schiff stain. While these histologic changes are consistent with the observed alterations in renal function, it is emphasized that the changes described are nonspecific and no extensive structural alteration was found. This study does not demonstrate permanent renal damage that can be ascribed to amphotericin B but it does suggest that recovery may not be as prompt or complete as previously believed. Our data show little or no correlation between the amount of drug given and the degree of dysfunction produced.

SUMMARY

Fifteen patients being treated with amphotericin B have had renal function studied by means of inulin and PAH clearances. These studies suggest that the altered renal function is due to diffuse changes in the kidney affecting not only glomerular filtration rate but also renal plasma flow. Renal biopsies obtained from three patients during the period of BUN elevation showed only slight, nonspecific histologic changes.

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DISCUSSION

DR. FURCOLOW: We have not been impressed with any persistent renal effect of amphotericin and the first question that would come to my mind is that since this drug is not very effective against coccidioidomycosis, what assurance is there that this 131 days and so forth after the beginning of treatment has anything to do with the drug rather than with the progression of the disease unaffected by the drug. The second is that our patients, whose average age is 65, would require very careful correlation of their tests before treatment in order to describe any great changes after treatment.

DR. RHOADES: In regard to the first question, the possibility that the altered function demonstrated after being taken off therapy might be due to progression of the disease, our experience with a relatively limited number of patients amphotericin B has been dramatic, even in coccidiodomycosis. I believe that this woman probably would have died without the drug and she has been followed for about a year and a half now. She had disseminated coccidioidomycosis cultured from the peritoneal cavity and other areas and I would expect that the improvement after this was due to therapy. We have limited data on people treated before the administration of amphotericin B so we can't actually say that the altered renal function certainly could not be due to the underlying disease. I think that's a valid criticism. In our laboratory we had considerable variation with the PAH but I don't think that our data would be explainable on that basis. Using 30 males, ranging from 25 to about 55 years, the inulin clearances were done and the mean value was 135 ml/min. The lower limits of normal that I showed were two standard deviations down and for PAH, with 661.5 plus or minus 99.9, these were well below two standard deviations down.

DR. KATZ: I think there is agreement that amphotericin B does affect renal function and there is some evidence that there are some anatomical changes in the kidney. I think there is no evidence, however, that these renal changes are of significance in the long haul and there appears to be complete reversal to normal. I don't know whether you have done repeat renal biopsies on the patients who had these changes.

DR. KIRBY: The thing that concerns me about the effect of amphotericin is the need for long term therapy. We have had cases very ill with acute disseminated coccidioidomycosis and have persisted with high doses of the drug despite a high BUN and with the administration of steroids have had the BUN fall and the patient improve. The problem is that if one stops amphotericin therapy after, say, four months, the incidence of relapse in such cases is high. In two of our patients that did relapse several months later and were retreated, the second time the rise in BUN and renal toxicity was much quicker and much more marked than it was the first time. I think it may well be in this disease, as with tuberculosis, that therapy really needs to be given for two years. It needs prolonged suppressive action and we have now been treating on an outpatient basis, with amphotericin, patients who come in three days a week. I would like to ask for any comments on this and express some concern about the renal effect of more prolonged therapy.

DR. RHOADES: We don't have any data on repeated therapy. We face exactly the same problem and we have people, for instance, with cryptococcal meningitis, in whom we have been able to demonstrate the organisms in the spinal fluid for months and months, even when we could not get it to grow, and we don't know quite where to stop. It is again like the treatment of tuberculosis. You never know when it's too long. You only know when it's too short. We have treated people on an outpatient basis three times a week. Also, we have considered giving people courses of therapy for three months and letting them off to see how they do. If they do poorly, we give them another course of therapy but we haven't gotten very far with that. I think the data I have presented today do indicate that the more severely ill patient, regardless of underlying disease, reacts in a more severe fashion to the drug. The two people who had mild reactions, that is as far as the BUN elevation is concerned, were young healthy men who had fairly localized disease and were treated with amphotericin, one because he had a lung resection and the other because he had isolated pulmonary cryptococcosis. I wonder if the studies that are done on drug toxicity in healthy medical students, for instance, can actually be applied to sick people, who, I'm convinced, react in a different way. But I don't know about quantitative studies in regards to linear treatment.

DR. FURCOLOW: I think there are two or three aspects to this thing. When you treat a lot of patients, and I think we have treated over 150 now with amphotericin, the renal toxicity is quite variable. Some patients show a marked rise early in the game which tapers off later. Some patients never show any rise and some of them have to have their dosage cut down if the NPN rises above 50. It's difficult to make generalizations. I'm sure Kirby is right about the prolonged treatment. We don't know yet how long you have to treat them, and coccidioidomycosis is complicated by the fact that apparently only one third to one half of the strains are sensitive to amphotericin, so that while you have resistant strains, you also have the problem of prolonged therapy because of dissemination of the disease. In histoplasmosis and blastomycosis, we have tested some 30 or 40 strains of histoplasma without finding any variation in the sensitivity to amphotericin. It does not appear that we have resistant strains. Even with amphotericin there is evidence again of differences in people with cavitory disease in the length of time that they must be treated in order to get what we would call a relatively permanent cure. I think all these problems need to be evaluated on more cases.

TETRACYCLINE PHOSPHATE COMPLEX AND AMPHOTERICIN B IN THE TREATMENT OF URINARY TRACT INFECTIONS*

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INTRODUCTION

The work of many investigators indicates beyond question that the tetracycline antibiotics, given orally, can have a marked effect upon the fecal flora of man [1] with resultant diarrhea, gassy distention, rectal pruritis, and burning. Control of the growth of intestinal yeasts with resultant reduction of gastrointestinal symptoms by the addition of nystatin to tetracycline has been well documented by a number of investigators [2-4]. Recently another antibiotic, amphotericin B, has been found to be effective in the prevention of antibiotic-induced moniliasis [5]. Our own previous experience with tetracycline phosphate complex in the treatment of urinary tract infections [6], however, did not reveal a high incidence of severe diarrheas allegedly due to fecal flora change.

This is a report on a series of 50 patients treated with capsules of tetracycline phosphate complex and an antifungal agent, amphotericin B.† Each capsule contained tetracycline phosphate complex equivalent to 250 mg tetracycline hydrochloride plus 50 mg amphotericin B. The purpose of this study was to observe its clinical and bacteriological effectiveness, patient tolerance, and emergence or lack of emergence of microorganisms not initially isolated prior to therapy.

PROCEDURE

A series of 50 patients, primarily ambulatory, with urinary tract infections, mostly chronic, associated with either Gram-positive or Gram-negative organisms, or both, was studied. These patients were selected on the basis of clinical symptoms such as dysuria, urgency, burning, and frequency and, in most instances, the presence of one or more organisms in the urine (catheterized specimen in the female or a clean, voided specimen in the male). Many patients had received no treatment for at least six months prior to this study. Those who had received some form of therapy had been treated with a sulfonamide drug with unsatisfactory response. Several patients were placed on this tetracycline combination regardless of demonstrated bacterial resistance of isolated organisms. Previous experience had shown that occasionally patients harboring organisms resistant to the tetracycline group manifested a favorable clinical response to the drug [6].

The arbitrary dosage of this drug was one capsule q.i.d. or a total of 40 capsules. In some instances a larger initial dosage was used for a few days where medical prudence seemed to indicate it (Table 1). Since the patients were

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*This investigation was supported by a grant from Squibb Institute for Medical Research, New Brunswick, New Jersey. The capsules were supplied by Dr. John T. Groel of Squibb Institute for Medical Research.

†The trade name of E. R. Squibb & Sons Division, Olin Mathieson Chemical Corporation, New Brunswick, New Jersey, for tetracycline phosphate complex (Sumycin) and amphotericin B (Fungizone) is Mysteclin-F.

Table 1. Mysteclin F – Capsules, Clinical and Bacteriological Response

Patient	Diagnosis	Organism	Tetracycline sensitivity*	Dosage†	Response		Side effect
					Clinical‡	Bacteriological	
G. A.	Urethrocystitis	*A. faecalis*	S	1 qid-10	Improved	*P. aeruginosa*	Nausea, followed by diarrhea.
		P. aeruginosa	R				
T. A.	Urethral stricture; low grade chronic urethrocystitis	*A. aerogenes*	S	1 qid-10	Improved	NI§	None
G. C.	Chronic interstitial cystitis	*A. faecalis*	S	1 qid-7	Improved	NI	None
S. C.	Chronic pyelonephritis	*A. aerogenes*	R	1 qid-10	Unchanged	*A. aerogenes*	Slight gassy indigestion after 7 days.
B. E.	Chronic cystitis	*A. aerogenes*	S	1 qid-10	Cured	NI	None
R. E.	Chronic nephrolithiasis with pyelonephritis	*E. coli*	S	1 qid-14	Improved		None
		P. aeruginosa	R			*P. aeruginosa*	
R. F.	Chronic urethroprostatitis	Enterococcus	S	1 qid-10	Improved	NI	Generalized aches persisted until medication stopped.
S. F.	Urethroprostatitis	*S. aureus*, coag.+	S	1 qid-7	Improved	NI	None
B. J.	Urethrocystitis with vaginitis	*S. aureus*, coag.–	S	1 qid-10	Improved	NI	Slight nausea
C. I.	Chronic urethrocystitis	*A. aerogenes*	R	1 qid-10	Cured	NI	None
C. J.	Urethrocystitis	*P. aeruginosa*	R	1 qid-16	Improved	*P. aeruginosa*	None
C. L.	Chronic pyelonephritis	*A. aerogenes*	R	1 qid-10	Improved	*A. aerogenes*	None
C. M.	Chronic cystitis	*A. aerogenes*	R	1 qid-7	Improved	*A. aerogenes*	None
E. O.	Urethrocystitis	*A. aerogenes*	R	2 tid-4 1 qid-10	Improved	*A. aerogenes*	Slight heartburn with indigestion.

Table 1 cont.

Patient	Diagnosis	Organism	Tetra-cycline sensi-tivity*	Dosage†	Response		Side effect
					Clinical‡	Bacteriological	
D. P.	Chronic cystitis with urethral stricture	No org.; pyuria	–	1 qid-10	Improved	NI; pyuria cleared	None
W. R.	Nonspecific urethritis;	*A. aerogenes*	S	1 qid-7	Improved	NI	None
C. W.	Subacute urinary tract infection	*E. coli*	S	1 qid-10	Improved	Enterococcus	Slight heartburn first 3 days; disappeared.
H. W.	Chronic urethrocystitis	*A. aerogenes*	R	1 qid-10	Cured	NI	None
P. W.	Acute hemorrhagic cystitis	No org.; pyuria	–	1 qid-7	Cured	NI; pyuria cleared	None
R. W.	Urethroprostatitis	*S. aureus*	S	1 qid-10	Cured	NI	None
J. C.	Urethrocystitis	Enterococcus	S	1 qid-7	Cured	NI	None
M. Co.	Chronic pyelonephritis; incipient uremia	*E. coli*	S	1 qid-7	Improved	*E. coli*	None
V. D.	Recurrent urethro-cystitis	No org.; pyuria	–	1 qid-7	Unchanged	Unchanged	Nausea after 7 days; therapy discontinued.
E. R.	Chronic nephrolithiasis; pyelonephritis	*E. coli* *P. aeruginosa*	S R	1 qid-14	Improved	*E. coli*	None
F. S.	Urethroprostatitis	No org.; pyuria	–	1 qid-10	Improved	NI; pyuria cleared	None
J. A.	Low grade pyelo-nephritis	No org.; pyuria	–	1 qid-7	Improved	NI; pyuria cleared	None
R. B.	Chronic cystitis with pseudomembranous trigonitis	No org.; pyuria	–	1 qid-8	Improved	NI; pyuria cleared	None
E. B.	Urethrocystitis	*A. aerogenes*	S	1 qid-10	Cured	NI	None
P. B.	Perineal abscess; chr. chronic prostatitis	No org.; pyuria	–	2 tid-7	Improved	NI; pyuria cleared	None

Table 1 cont.

Patient	Diagnosis	Organism	Tetracycline sensitivity*	Dosage†	Response		Side effect
					Clinical‡	Bacteriological	
J. B.	Urethrocystitis; vaginitis	α-hemo. Streptococcus, *S. albus*	– S	1 qid-10	Improved	NI	Slight Nausea
J. Ch.	Chronic urethrocystitis; urethral stricture	*A. faecalis* *A. aerogenes*	S R	1 qid-10	Cured	NI	None
M. C.	Chronic cystitis; hypertonic neurogenic bladder	*A. aerogenes*	R	1 qid-7	Improved	No change	None
L. C.	Chronic pyelonephritis; lower ureteral stricture	No org.; pyuria	–	1 qid-7	Cured	NI; pyuria cleared	None
J. S.	Benign prostatic hypertrophy with urethroprostatitis	No org.; pyuria	–	1 qid-7	Cured	NI; pyuria cleared	None
G. D.	Cancer of prostate with chronic urethrocystitis	*S. aureus*, coag.– Enterococcus yeasts	R S	1 qid-14	Improved	NI	None
B. K.	Chronic cystitis	*E. coli*	R	1 tid-14	Cured	NI	None
J. Cr.	Urethrocystitis with large caliber urethral stricture	*A. aerogenes* *P. aeruginosa*	R R	1 qid-14	Slightly improved	*A. aerogenes* R Enterococcus R *A. faecalis* S *G. tetragenes* R	None
P. D.	Chronic cystitis; urethral stricture	No org.; pyuria	–	1 tid-14 1 bid-18	Improved	NI; pyuria cleared	None
O. E.	Urethrocystitis; post-superpubic prostatectomy	*A. aerogenes*	R	2.tid-3 1 qid-10	Improved	No change	Slight heartburn and indigestion.

Table 1 cont.

Patient	Diagnosis	Organism	Tetracycline sensitivity*	Dosage†	Response		Side effect
					Clinical‡	Bacteriological	
A. G.	Urethrocystitis	Non-hemo. Strep. A. faecalis P. aeruginosa Gaffkya tetragena	 S R R	1 qid-7	Improved	No change	Nausea, diarrhea
C. G.	Chronic interstitial cystitis	A. faecalis	S	1 qid-5	Improved	No change	None
W. H.	Chronic urethrocystitis; urethral stricture	A. aerogenes P. aeruginosa	R R	1 qid-14	Cured	NI	None
W. M.	Subacute urinary tract infection	E. coli Enterococcus	S R	1 qid-7	Slightly improved	A. faecalis S G. tetragenes R	Slight heartburn when taken with meals only.
W. P.	Acute hemorrhagic cystitis	No org.; pyuria	—	1 qid-5	Improved	NI; pyuria cleared	None
F. R.	Chronic urethro-prostatitis	No org.; pyuria	—	1 qid-10	Improved	NI; pyuria cleared	Generalized aching.
W. R.	Urethroprostatitis with hemospermia	S. aureus Enterococcus	S S	1 qid-4 1 tid-10	Cured	NI	None
R. S.	Urinary tract infection; pharyngitis	A. aerogenes Yeasts	S	1 qid-10	Unchanged	Yeasts Enterococcus R A. faecalis S	Slight gaseous indigestion.
G. T.	Chronic cystitis	E. coli	R	1 qid-4	Slightly improved	Unchanged	Anorexia and nausea with diarrhea.
A. T.	Chronic urethrocystitis; urethral stricture	No org.; pyuria	—	1 qid-7	Improved	NI; pyuria cleared	None
R. W.	Nonspecific vaginitis; urethritis	No org.; pyuria	—	1 qid-5	Cured	NI; pyuria cleared	None

*S = Sensitive; R=Resistant, by agar-diffusion technique to 30μg Difco tetracycline disk.

†Number of capsules, frequency, number of days' duration.

‡Cured=Pyuria and symptoms eliminated; Improved=Pyuria and symptoms markedly reduced.

§No organism isolated

primarily private cases and ambulatory, it was not always expedient to obtain urine specimens at stated intervals for culture, particularly after cessation of therapy. In several instances when, on microscopic examination of the urine, pyuria was absent together with the absence of symptoms, it was felt unnecessary to submit the patient to catheterization in order to obtain a specimen for bacteriological examination. Such patients were presumed to be cured clinically.

Certain terms used in this report are defined as follows: Cured is used to signify elimination of clinical symptoms together with elimination of pyuria whether or not all bacteria have been eliminated; improved refers to a marked reduction but not elimination of clinical symptoms, together with a marked reduction of pyuria with or without sterilization of the urine; unchanged refers to no symptomatic relief following therapy with the microorganisms usually persisting.

RESULTS

Table 1 presents a composite picture of the patient identification, the clinical diagnosis, the incriminating organism or organisms when present, bacterial susceptibility, dosage, clinical response, bacteriological response, and side effects. Of the 50 patients treated with this drug, 14 were considered cured clinically. All 14 showed simultaneous elimination of microorganisms although four of these had no organism isolated initially and were treated solely on the basis of symptoms and pyuria. It is interesting to note that of the remaining ten, while five showed organisms susceptible to tetracycline initially, four others demonstrated resistant Aerobacter aerogenes (one also combined with a resistant Pseudomonas aeruginosa) and one had had a resistant Escherichia coli.

Of these 50 cases, 33 were considered improved in that there was a marked improvement symptomatically with a marked reduction in pyuria. The organism was apparently eliminated following cessation of treatment in 17 of these 33 cases. Nine of these 17 cases, however, were treated solely on the basis of pyuria and no organism was isolated initially or subsequently. All but one of the remaining eight showed susceptible organisms initially. The one resistant organism was a coagulase-negative Staphylococcus aureus. Of these 33 improved cases, 16, although clinically improved, still maintained the presence of one or more organisms following therapy. A resistant A. aerogenes persisted in six of these, a resistant P. aeruginosa in four, and the original organisms were eliminated but others emerged in two. One of these had had a resistant enterococcus initially. In the remaining four, one resistant E. coli, two susceptible E. coli, and one susceptible Alkaligenes faecalis persisted. Observation for the presence of the organism was usually made within one month following cessation of therapy.

Only three of the 50 patients showed no evidence of clinical improvement as a result of therapy. In one case the original organism, A. aerogenes, which was susceptible to tetracycline, was eliminated but a resistant enterococcus and an A. faecalis emerged while an existing yeast persisted. In the second, a resistant A. aerogenes persisted and the third was treated solely on the basis of pyuria, which did not clear.

In only two patients were yeasts found initially. These persisted following therapy in one case and were eliminated in the other case. In neither case was it felt that they were clinically significant or directly associated with the urinary tract infection. They were not identified with Monilia. No yeasts not existing initially were found in any subsequent specimens.

SIDE EFFECTS

In 36 of the 50 cases treated with this drug as previously described (Table 1) there were no side effects of any nature. Of the remaining 14 patients, one showed nausea severe enough to discontinue therapy after 7 days; three showed diarrhea with accompanying nausea; four demonstrated slight heartburn with indigestion (although in one of these the symptoms subsided when the patient took the capsule after eating instead of during the meal); two demonstrated gaseous indigestion, two slight nausea, and two complained of generalized aching. Side effects, when they did occur, usually started after the fifth day of therapy.

Side effects in general were mild. In only one instance, in which nausea was experienced, were they severe enough to interrupt treatment. As observed with tetracycline phosphate complex alone [6], the outstanding observation was the lack of severity of diarrhea as compared with tetracycline hydrochloride. It is interesting to note that the few cases of sore tongue encountered with tetracycline-phosphate-complex therapy were not observed in this series. Stone and Mersheimer [4] also noted the absence of glossitis (found in patients treated with tetracycline alone) when nystatin was added to the tetracycline. Since glossitis is often due to candidiasis, it may well be that changes produced in the oral flora by tetracycline allowing emergence of Candida may have been overcome by the addition of amphotericin B.

If all side effects are considered, even the mildest and most temporary, then the over-all incidence is comparable to that encountered with tetracycline hydrochloride but the severity is strikingly less.

DISCUSSION

Present and past experiences have shown that "observed differences between tetracycline preparations are largely of academic interest" [7] and have little, if any, significance in terms of therapeutic response. However, efficacy can be a function of patient tolerance. The tetracycline phosphate complex and the antifungal agent apparently improve patient tolerance, thus allowing completion of the full course of therapy. This more often resulted in a higher percentage of cures. As far as patient tolerance is concerned, our experience indicates that this drug is superior to many of the tetracyclines previously tried. The lessened severity of side effects was striking.

The duration of therapy with this drug averaged 10 days although one individual responded after 4 days and another took 32 days (Table 1). The usual total consumption of capsules varied between 35 and 39. There is no definitive evidence to indicate that a specific number of capsules is necessary for optimum response on a routine basis. This is a matter for the clinician to decide. Otherwise, a regimen probably should be set arbitrarily at one capsule (equivalent to 250 mg tetracycline hydrochloride) q.i.d. for 10 days.

Complete, permanent cure in chronic urinary tract infections, regardless of therapy, is rare, so that the best one can hope for is temporary cure or improvement (for from 1 to 12 months). In only about 60% of these patients was the organism apparently eliminated even though there was evidence of clinical improvement. This observation is comparable to that made with other tetracycline preparations. This series of cases did not indicate that this drug had any unusual affinity for a given organism which would render it superior to the other tetracyclines from an antibacterial standpoint. The incidence of yeasts in this series of chronic urinary tract infections was so low that no evaluation can be placed on the efficacy of this drug against them. If one combines the number of patients who were considered clinically cured with those who showed clinical improvement, one finds that this drug demonstrates an efficacy comparable to the best of any of the other tetracycline preparations.

CONCLUSIONS

At the present time it is felt that, because of its high degree of tolerability and the notable lack of severe diarrhea in connection with its use, tetracycline phosphate complex with amphotericin B is to be considered a preferred tetracycline drug in the treatment of most chronic urinary tract infections when a tetracycline drug is indicated.

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DISCUSSION

DR. KIRBY: The observation here, as I understand it, is that by combining the amphotericin and tetracycline the incidence of diarrhea is increased. Is that right?

DR. TRAFTON: No, decreased.

DR. KIRBY: I think this is reminiscent of the studies that have been reported previously in these meetings with other antifungal agents where, as I recall, Dr. Hewitt of Los Angeles showed in crossover studies with controls that there was no difference in the incidence of diarrhea whether one did or did not use the antifungal treatment. I would just like to ask if there are such controls in this series and what the results would have been under these circumstances.

DR. TRAFTON: We ran no concurrent controls but this evidence was based on other papers that have been given during the past five or six years on, I suppose, every tetracycline that has come along. Except for demethylchlortetracycline, which I reported on last year, this has been the lowest incidence of diarrhea, although we did run into other troubles with it. But if we exclude DMCT we have the lowest incidence of diarrhea in this particular preparation of any that I have worked on before.

DR. KATZ: I'm sure Dr. Kirby will find that an unacceptable answer and I don't blame him. In using the preparation of tetracycline with amphotericin in the therapy of pneumonia and alternating cases, we found no difference in the incidence of side effects. I think the mere presence of Candida in the stools has no meaning whatsoever. We certainly found no difference of any significance with this preparation.

TOXICITY AND ABSORPTION STUDIES IN HUMANS OF NEWER ANTIFUNGAL AGENTS

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INTRODUCTION

With increasing recognition of the problem of severe systemic mycotic infections in large areas of this country there has been a continued stimulus to the search for effective antifungal agents. In a continuing study conducted in our laboratory more than 100 substances have been tested for antifungal activity. In a companion study, the Communicable Disease Center Cooperative Histoplasmosis Study, clinical material and data have been made available from hospitals and sanatoria on a large number of proven cases of systemic mycotic infection. The availability of both laboratory facilities and proven cases has enabled us to undertake detailed toxicity and absorption studies on compounds which appear to be promising as antifungal agents. The present report summarizes both the in vitro sensitivity studies and the toxicity and absorption data on a group of newer antifungal agents.

MATERIALS AND METHODS

The tissue-culture method of Larsh et al. [1] was used in screening all substances for antifungal activity. The initial procedure in each case consisted of testing four tenfold dilutions (100, 10, 1, 0.1 μg/ml) of the substance against five test organisms, Histoplasma capsulatum, Blastomyces dermatitidis, Cryptococcus neoformans, Sporotrichum schenckii, and Candida albicans. Suitable organism, media, and solvent controls were included with each test. Activity levels in terms of 50% inhibition of growth were then calculated for each substance. Furthermore, those substances demonstrating significant inhibitory effect (50% inhibition at 10 μg/ml or less) were tested by intermediate dilutions to confirm the calculated 50% inhibition level and to pinpoint the level at which complete inhibition might occur.

Arbitrarily, the 50% inhibition level of 10 μg/ml or less was chosen to select those compounds which were to be considered for further studies. However, other factors including availability, stability, purity, and toxicity in animals ultimately determined the suitability of the antifungal compounds for toxicity and absorption studies in human cases. The studies were eventually performed in a group of 64 patients with deep mycotic infections. This group included 50 cases of histoplasmosis, seven cases of blastomycosis, four cases of cryptococcosis, and three cases of coccidioidomycosis. All cases were hospitalized during the period of testing and observation. In addition to the usual clinical and laboratory studies, toxicity was evaluated by blood counts and liver and renal function tests performed periodically according to a protocol.

Table 1. Concentrations, μg/ml, Producing 50% Inhibition Against Test Organisms of 38 Agents Demonstrating 50% Inhibition Against the Yeast Phase of *Histoplasma capsulatum* at 10 μg/ml or Less

Agent	Manufacturer	*Histoplasma capsulatum*	*Blastomyces dermatitidis*	*Cryptococcus neoformans*	*Sporotrichum schenckii*	*Candida albicans*
A 272	Lilly	3.2	1.0	3.8	0.46	>100.0
Amebacide 16726	Lilly	8.0	21.0	4.6	3.1	>100.0
Aminostilbamidine	Merrell	10.0	<0.1	4.6	1.0	31.0
Amphotericin B	Squibb	0.05	0.2	0.2	0.17	0.1
Anisomycin	Pfizer	<0.5	0.3	0.04	3.9	0.5
Antabuse	Ayerst	1.0	31.0	10.0	0.1	>100.0
Candicidin	S. B. Penick	0.09	<0.1	0.8	<0.1	<0.1
Dequadin acetate	Johnson & Johnson	0.8	0.8	0.5	0.5	1.0
Endomycin	Upjohn	0.43	21.0	10.0	0.1	31.0
Eulicin	Sharp & Dohme	0.43	<0.1	0.25	21.0	2.1
Hydroxystilbamidine	Merrell	1.0	1.0	10.0	100.0	31.0
L 509	Warner-Chilcott	3.2	10.0	4.6	1.0	30.0
Lilly 13776	Lilly	3.8	3.8	3.8	32.0	3.8
Lilly 16728	Lilly	3.8	3.8	3.8	1.0	3.8
Lilly 23605	Lilly	0.46	1.0	1.0	3.8	4.6
Lilly 25948	Lilly	3.2	3.2	0.46	0.32	3.2
Lilly 26882	Lilly	1.0	2.2	4.3	10.0	31.0

Table 1 cont.

Agent	Manufacturer	*Histoplasma capsulatum*	*Blastomyces dermatitidis*	*Cryptococcus neoformans*	*Sporotrichum schenckii*	*Candida albicans*
Lilly 25918	Lilly	0.38	0.38	1.0	1.0	1.0
Lilly 25947	Lilly	2.8	2.2	1.2	1.5	5.0
Lilly 28440	Lilly	0.46	0.46	1.0	0.32	3.8
Lilly 29348	Lilly	4.6	4.6	46.0	1.0	38.0
Lilly 33432	Lilly	1.0	4.6	<1.0	<1.0	4.6
M & B 938	May & Baker	0.1	1.0	1.0	3.0	10.0
MRD-112	Merrell	10.0	3.2	1.8	21.0	>100.0
Mycostatin	Squibb	3.2	3.2	3.2	2.1	3.2
Nepera 1112	Nepera	1.1	3.2	2.7	3.2	32.0
Nepera 1968	Nepera	1.3	3.2	1.0	1.7	3.2
P A 153	Pfizer	3.8	2.5	2.5	3.8	3.8
P A 166	Pfizer	3.8	2.5	3.8	3.8	4.6
Pimaricine	Lilly	3.8	3.2	3.8	4.6	3.8
Pyridium chloride	Johnson & Johnson	4.6	4.6	10.0	1.0	>100.0
R O 2-7758	Hoffman La Roche	2.2	0.22	>100.0	>100.0	>100.0
S D 7088	Shell Chemical	3.2	32.0	38.0	32.0	>100.0
Trichomycin	Fujisawa	<0.1	<0.1	0.44	0.32	0.22
U-0178	Upjohn	4.6	38.0	38.0	38.0	38.0
U-8869	Upjohn	1.0	0.1	0.46	0.46	46.0
U-9715	Upjohn	3.2	3.2	4.6	46.0	32.0
Variotin	Lederle	5.0	19.0	5.0	23.0	160

Gastrointestinal absorption of the oral agents was evaluated by controlled serum-level determinations. Bleedings were taken at intervals (usually 1, 2, 3, 6, 12, and 24 hr) after administration of the various agents. The procedure for serum-level determinations consisted of making serial dilutions of the serum in HeLa maintenance medium contained in tissue-culture tubes. A stock strain (No. 1539)* of C. albicans was used as the test organism in the serum-level determinations of those agents which had demonstrated a level of 50% inhibition against that organism in the range of 1.0 μg/ml or less. Using this organism, the results of the test are easily read at 24 hr. However, in some cases the agent being studied for absorption was significantly less active in vitro against C. albicans than against the yeast phase of H. capsulatum. With these latter agents, the more slowly growing (usually 72 hr for 4+ growth in the controls) Histoplasma yeast had to be utilized as the test organism. The prolonged incubation at 37 C may allow a variable degree of inactivation or decomposition of the agent before inhibition can be evaluated [2].

RESULTS

Of the more than 100 substances tested to date, 38 have been found which demonstrated 50% inhibition levels against H. capsulatum of 10 μg/ml or less. Table 1 lists these 38 agents and the concentrations at which they produce 50% inhibition against the test organisms.

Because of the limiting factors mentioned previously, only 13 of these agents were considered suitable for clinical studies in human cases. Table 2 lists these 13 agents and their routes of administration.

Table 2. Antifungal Agents Used in Clinical Trials

Antibiotics	Route	Chemicals	Route
Amphotericin B	I.V.	MRD-112	Oral
Amphotericin B	Oral	Stilbamidine	I.V.
Mycostatin	I.V.	Hydroxystilbamidine	I.V.
Trichomycin	Oral	Disulfiram	Oral
Pimaricin	Oral	Amebacide	Oral
		Upjohn U-0178	Oral
		Lilly 25948	Oral
		May & Baker 938	Oral

The intravenous form of amphotericin B was one of the agents evaluated early in the course of this study and its effectiveness is presently under extensive evaluation by the Communicable Disease Center Cooperative Histoplasmosis Study in a large series of proven systemic mycotic infections which includes more than 100 cases of chronic pulmonary histoplasmosis alone. At present it is the only drug being used by this group and has been established as the therapeutic standard against which all future agents will be compared. However, this discussion is limited to the 12 agents other than intravenous amphotericin B.

Three intravenous agents, mycostatin, stilbamidine, and hydroxystilbamidine were among the group tested for toxicity and possible therapeutic effect. In ten cases of histoplasmosis no therapeutic effect was noted with any of the agents [3] and, although four cases of blastomycosis appeared to respond favorably to hydroxystilbamidine, difficulties in administration and potential toxicity appeared to limit its usefulness.

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*Kindly supplied by the Squibb Institute for Medical Research, New Brunswick, New Jersey.

Since the necessity for repeated and often prolonged injections of intravenous agents has placed obvious limitations on their acceptability, the major efforts throughout the remainder of the study were directed towards the evaluation of oral agents.

Three of the nine oral agents evaluated were antibiotics and six were synthetic chemicals. Table 3 summarizes the dosages used and the side effects noted with each agent. Only one of the antibiotics, amphotericin B, and three of the chemicals, MRD-112, disulfiram, and amebacide, did not cause significant gastrointestinal side effects. With the other agents these effects were usually so severe that continuous dosage could not be tolerated for more than 1 to 7 days.

Table 3. Oral Antifungal Agents Used in Clinical Trials

Agent	Daily oral dosage	Side effects
Amphotericin B		
Insoluble form	2.0 to 7 g	None
Solubilized form	0.5 to 2 g	None
Trichomycin	50,000 to 1,000,000 units	Cramping, diarrhea
Pimaricin	125 to 500 mg	Severe cramping, diarrhea
MRD-112	0.5 to 2.0 g	None
Disulfiram	0.5 g	None
Amebacide	45 to 90 mg	None
Upjohn U-0178	0.2 to 2.0 g	Moderate nausea, diarrhea
Lilly 25948	0.2 to 2.0 g	Moderate nausea, diarrhea
May & Baker 938	0.25 to 1.0 g	Severe cramping, diarrhea

A total of 55 serum-level determinations were performed on 37 patients who received six of the oral agents. Table 4 summarizes the results obtained in these determinations. Diamidinodiphenylamine dihydrochloride (M & B 938) was the only compound evidencing any gastrointestinal absorption following single and repeated dosage. However, following a single 250-mg dose of this drug (packed in a gel capsule), seven of the eight patients experienced severe abdominal cramping and diarrhea and only one was able to tolerate repeated doses for longer than 7 days. Despite this marked purgative effect, significant peak serum concentrations ranging from 0.4 to greater than 6.7 μg/ml were demonstrated in five 24-hr absorption tests conducted in four patients. The variation in the peak levels observed and the apparent lack of absorption of this drug in four other patients receiving the same dosage may be related in part to its irritant effect on the gastrointestinal tract. One patient was able to tolerate M & B 938 in a dosage of 250 mg every 12 hr for 13 days. In this patient, though the initial single dose gave a peak level of 3.6 μg/ml at 2 hr, there appeared to be an increase in absorption and accumulation of the drug following the interval of repeated dosage since 14 days later a peak level of more than 6.7 μg/ml was obtained at 6 hr and a significant level (0.4 μg/ml) was detected 24 hr after a single 250-mg dose.

Table 4. Oral Antifungal Agents, Results of Sensitivity and Serum Level Determinations

Drug and manufacturer	Sensitivity[*]		Serum level[*]		
	H. capsulatum	*C. albicans*	Patients tested	Number of tests	Number with + level
Amphotericin B Squibb	<0.05	<0.1[†]	5	7	0
Trichomycin Fujisawa	<0.1	0.22[†]	3	4	0
Pimaricin Lilly	3.8	3.8[†]	8	11	0
U-0178 Upjohn	4.8[†]	38.0	6	11	0
29548 Lilly	3.2	3.2[†]	7	13	0
Diamidinodiphenylamine dihydrochloride M & B 938 May & Baker	0.1[†]	10.0	8	9	5

[*] 50% inhibition, μg/ml

[†] Denotes organism used in serum-level assay.

Because of the significant serum levels obtained after oral administration of diamidinodiphenylamine, the drug was administered over prolonged periods (29 to 60 days) to three patients with chronic cavitary histoplasmosis who were able to tolerate doses gradually increased to daily totals of 250 to 750 mg. With these doses, serum levels comparable to those demonstrated previously were obtained but there was no apparent clinical or x-ray improvement and sputum cultures remained positive for *H. capsulatum* throughout the course of treatment in all three cases. Furthermore, though none of the three patients developed clinical jaundice or hepatomegaly, decreased plasma-prothrombin levels (approximately 70% reduction) were observed in all three and gradual elevations of serum glutamic pyruvic transaminase to 117 units (normal = < 40 units) were observed in one case. Both prothrombin and transaminase levels returned to normal following cessation of the drug. Serum-bilirubin and cephalin-flocculation values were not affected by the drug.

SUMMARY

In the continued search for effective antifungal agents a total of 38 substances with 50% inhibition of *Histoplasma capsulatum* at levels of 10 μg/ml or less have been found. Thirteen of these antibiotics and chemicals were found suitable for toxicity and absorption studies in humans. Three intravenous preparations, mycostatin, stilbamidine, and hydroxystilbamidine, were inferior to amphotericin B administered by the same route. Of the nine oral drugs only MRD-112, amebacide, disulfiram, and amphotericin B did not produce significant gastrointestinal side effects.

Of the oral drugs tested in 49 individuals, only diamidinodiphenylamine dihydrochloride gave evidence of absorption by serum-level determinations. Though the results of absorption studies with this drug were not uniform, probably due to gastrointestinal irritation, four of eight patients receiving the drug demonstrated peak levels of 0.4 to 3.9 μg/ml 3 to 12 hr after a single 250-mg dose. In the one patient who tolerated repeated dosage there was evidence of increased absorption and accumulation of the drug after 13 days of repeated dosage. More prolonged daily dosage (250 to 750 mg for up to 60 days) in three histoplasmosis cases produced significant hepatic toxicity as indicated by elevation of transaminase levels in one case and depressions of plasma-prothrombin activity in all three. These abnormalities proved reversible following cessation of the drug. Clinical, x-ray, or cultural improvement could not be demonstrated in any of the patients who received oral diamidinodiphenylamine.

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DISCUSSION

DR. KATZ: I'd like to ask Dr. Lynch, what was the largest amount of amphotericin given by mouth and the blood-level data obtained.

DR. LYNCH: I'm fairly sure that in one of our cases that I wasn't able to verify when I was writing this up, more than 7 g/day were given. However, as far as I know, of the ones that had blood-level studies, 7 g was the highest dose of the oral preparation. We have tried the intravenous and capsule forms also, as you know, but we have not pushed it to that high a level.

DR. KATZ: Every once in a while we read reports of the therapy of systemic fungal infections with oral amphotericin and one often wonders what is being accomplished when this is done. In reports in the literature there are some in which large amounts of amphotericin were given and levels as high as 3 gamma were obtained but I think your work ought to close the book on use of oral amphotericin in treatment of systemic fungal infection.

ACTION OF POLYENE ANTIBIOTICS ON CANDIDA*

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INTRODUCTION

A decade ago isolation of an antibiotic possessing broad antifungal activity was announced at the autumn meeting of the National Academy of Sciences in Schenectady, New York [1]. The antibiotic, named nystatin subsequently, contained a characteristic series of carbon-carbon double bonds in conjugated linkage. This feature of the molecule was the basis for designating nystatin and related antibiotics as polyenes [2]. In addition, nystatin, amphotericin B, and pimaricin have been found to contain an amino-sugar moiety, mycosamine [3]. The structural formula of pimaricin, the first polyene whose structure was elucidated, is shown in Fig. 1 [4]. Since the discovery of nystatin, several polyenes have been isolated from streptomycete beers. Indeed, almost every soil sample has yielded a streptomycete capable of producing at least one member of this family of antibiotics [5]. Of these, only amphotericin and nystatin enjoy widespread use although clinical trials with pimaricin [6], in vitro studies with endomycin [7], and field tests with filipin [8] are still in progress.

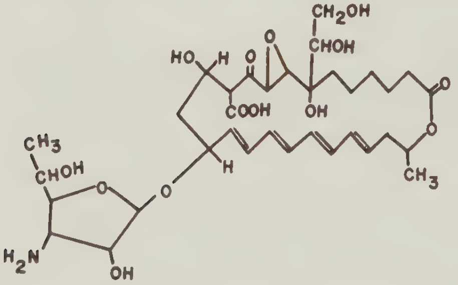

Fig. 1. Structural formula of the tetraene pimaricin (after Patrick et al., 1958).

Our interest in these compounds stemmed from attempts to prevent proliferation of Candida stellatoidea without impairing certain metabolic activities [9]. During the course of these investigations, our attention has become focused on the mechanisms of action of the polyene antibiotics, first on nystatin and subsequently on pimaricin, filipin, endomycin, and amphotericins A and B. This report presents evidence that the modes of action of the different polyenes are fundamentally the same and that the primary, vulnerable, physiological locus is the cellular permeation system.

MATERIALS AND METHODS

C. stellatoidea strain CS and a mutant derived from it by direct selection were used throughout this investigation. The nystatin-resistant variant could

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*This investigation was supported by a grant-in-aid of research from the Brown-Hazen Fund of the Research Corporation.

grow in medium containing 20 μg antibiotic per milliliter, whereas the parental strain was unable to grow in the presence of 1 μg/ml. Stock cultures were maintained on growth medium composed of 20 g glucose, 1 g Difco yeast extract, 1 g NH_4Cl, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 15 g Difco agar, and 1 liter deionized water. Quantitative growth experiments were carried out in this medium devoid of agar. Growth was measured turbidimetrically with a Klett-Summerson photoelectric colorimeter using filter No. 42 and by counting samples of yeast suspensions with the aid of a hemocytometer. Counts were made at a magnification of 860 diameters. A yeast cell with a distinct bud was scored as two units.

The antibiotics used were amphotericins A and B (Squibb), which were supplied by Dr. J. D. Dutcher, endomycin (Upjohn) and filipin (Upjohn), supplied by Dr. Bruce Churchill, pimaricin (Lederle), supplied by Dr. J. A. McMillen, nystatin (Squibb), cycloheximide (Upjohn), and griseofulvin (Ayerst). The polyenes were dispersed in absolute methanol at a concentration of 5 mg/ml. Only filipin and endomycin were completely soluble under these conditions. Stock reagents were stored at −20 C and were prepared anew monthly. Dilutions of drugs were made, as indicated, in water, in 0.067 M NaH_2PO_4 at pH 5 or in growth medium. In growth studies antibiotic was added to cool, sterile medium immediately before it was used. Manometric experiments conformed to the protocol adopted in earlier work [10].

RESULTS

C. stellatoidea strain CS was very susceptible to the action of polyene antibiotics; as little as 0.5 μg/ml amphotericin A, amphotericin B, nystatin, or filipin retarded growth measurably and 1 μg/ml suppressed growth effectively. Endomycin and pimaricin were less potent (Table 1). Minimal inhibitory concentrations of these antibiotics permitted the population of viable cells to remain constant for 24 hr if pH 5 growth medium was seeded with $5 \cdot 10^5$ yeasts per milliliter and incubated at 30 C (Table 2). Suppression of growth by filipin, pimaricin, nystatin, and amphotericin B but not by endomycin and amphotericin A, was negated by simultaneous addition of 100-500 μg cysteine per milliliter of medium. The protection was not related directly to the sulfhydryl group because ascorbate also lessened nystatin action (Table 3). It was not necessary to present the antibiotic and the reducing compound at the same time; for example, cysteine added 24 hr after exposure of the inoculum to repressive doses of nystatin permitted the culture to develop. These results emphasized that at low concentrations polyenes either suppressed growth or allowed for balanced growth and death. The action of cysteine, therefore, might have been upon the drug itself

Table 1. Effect of Polyenes on Growth of *Candida stellatoidea*

μg/ml	Turbidity					
	Amphotericin A	Amphotericin B	Endomycin	Filipin	Nystatin	Pimaricin
0.0	600	600	600	600	600	600
0.5	125	510	545	475	450	600
1.0	0	65	490	0	0	525
2.0	0	0	135	0	0	285
3.0	0	0	0	0	0	0
4.0	0	0	0	0	0	0

Cultures were seeded with $5 \cdot 10^5$ yeast cells per milliliter and incubated in aerated broth medium for 24 hr at 30 C. Turbidity was measured with a Klett-Summerson photoelectric colorimeter, filter No. 42.

Table 2. Survival of Candida in Growth Medium Plus Polyene

Antibiotic	Concentration, μg/ml	Survivors	Percent survivors
Amphotericin A	0.75	$4.4 \cdot 10^5$	88
	1.00	$7.5 \cdot 10^4$	15
	1.50	$3 \cdot 10^4$	6
Amphotericin B	1.50	$4.3 \cdot 10^5$	86
	2.00	$6 \cdot 10^4$	12
Endomycin	2.50	$4.7 \cdot 10^5$	94
	3.00	$2.5 \cdot 10^4$	5
Filipin	1.00	$5.1 \cdot 10^5$	102
	1.50	$3.5 \cdot 10^4$	7
	2.00	$5 \cdot 10^3$	1
Nystatin	0.75	$5.6 \cdot 10^5$	112
	1.00	$4.4 \cdot 10^5$	88
	1.50	$1.2 \cdot 10^5$	25
Pimaricin	2.50	$5.4 \cdot 10^5$	108
	3.00	$3 \cdot 10^5$	60
	5.00	$2 \cdot 10^4$	4

Growth medium was seeded to give $5 \cdot 10^5$ yeast cells per milliliter; cultures were incubated with shaking at 30C for 24 hr. Viable counts were made on growth medium agar; colonies were scored after 72 hr incubation.

rather than upon the damaged cells. Indeed, Candida cells exposed to deleterious concentrations of nystatin were altered irreversibly. Treatment of sensitive yeasts with 2 μg/ml for 12 hr or 5 μg/ml for 3 hr reduced the number of viable organisms significantly and addition of cysteine to the assay medium did not increase the ability of the injured population to form colonies (Table 4).

Polyenes affected fermentative and oxidative utilization of glucose. Of the antibiotics tested here, nystatin was the most effective; as little as 5 μg/ml reduced anaerobic carbon dioxide evolution substantially. At this drug concentration amphotericin B and endomycin stimulated gas production. At 25 μg/ml amphotericin A, amphotericin B, filipin, nystatin, and pimaricin inhibited glycolysis, whereas 50 μg endomycin per milliliter was required to impair fermentative capability (Table 5). The severity of suppression of glucose degradation was dependent upon pH and cation content of the environment. In 0.067 M potassium acetate, 4 μg nystatin per milliliter had scant effect on carbon dioxide production but in sodium acetate, glucose utilization was inhibited markedly. Under these conditions the action of the antibiotic was strongest at pH 6 and 7 (Fig. 2). Retardation by nystatin of fermentative catabolism of glucose by the drug-resistant Candida strain and the parental strain in 0.067 M sodium acetate was measured. Nystatin at 4 μg/ml inhibited glycolysis by both the sensitive and the resistant strains. The mutant was most susceptible at pH 5, whereas the wild type was suppressed most severely at pH 6. A very obvious difference between the two strains was the greater fermentative activity of the variant, contrasted to the parental strain, at the same optical density (Fig. 3).

Minimal growth-inhibitory levels of nystatin did not affect oxygen consumption by Candida when sodium lactate, sodium glutamate, sodium succinate, or glucose was the substrate. Respiratory utilization of sodium gluconate was stimulated by low levels of the drug. It is significant that normal cells could not use exogenous citrate but that nystatin-treated organisms did (Table 6). At this stage

Table 3. Effect of Cysteine and Ascorbate on Growth-Inhibiting Action of Polyenes

Polyene	μg/ml	Cysteine, μg/ml	Ascorbate, μg/ml	Growth
Amphotericin A	0.5	200	0	0
	0.5	0	200	0
	0.5	0	0	0
	1.0	0	500	0
	1.0	100	0	0
	1.0	500	0	0
	1.0	0	0	0
Amphotericin B	1.0	0	200	65
	1.0	100	0	240
	1.0	200	0	240
	1.0	0	0	65
	2.0	500	0	245
	2.0	0	0	0
Endomycin	2.0	200	0	105
	2.0	0	200	110
	2.0	0	0	105
	3.0	500	0	0
	3.0	0	0	0
Filipin	1.0	100	0	35
	1.0	200	0	145
	1.0	500	0	0
	1.0	0	200	75
	1.0	0	0	0
	2.0	500	0	0
	2.0	0	0	0
Nystatin	1.0	100	0	190
	1.0	200	0	210
	1.0	500	0	230
	1.0	0	200	0
	1.0	0	500	165
	1.0	0	0	0
	2.0	500	0	0
	2.0	0	0	0
Pimaricin	3.0	200	0	205
	3.0	500	0	0
	3.0	0	200	0
	3.0	0	500	0
	3.0	0	0	0
None	0	0	0	275
	0	100	0	250
	0	200	0	295
	0	500	0	305
	0	0	200	280
	0	0	500	265

Polyene, cysteine, or sodium ascorbate, when indicated, was added to sterile, cool medium prior to inoculation. Cultures were seeded with $5 \cdot 10^5$ yeast cells per milliliter and incubated in stationary broth medium for 18 hr at 30 C. Growth was measured with a Klett-Summerson photoelectric colorimeter, filter No. 42.

Table 4. Failure of Cysteine to Reverse Nystatin Damage

Nystatin concentration	Exposure, hr	Viable cells per milliliter		
		Initial	After treatment	
			+ Cysteine	Control
1	12	$13 \cdot 10^5$	$10 \cdot 10^5$	$11 \cdot 10^5$
1	24	$13 \cdot 10^5$	$12 \cdot 10^5$	$12 \cdot 10^5$
2	6	$14 \cdot 10^5$	$14 \cdot 10^5$	$13 \cdot 10^5$
2	12	$14 \cdot 10^5$	$6 \cdot 10^5$	$6 \cdot 10^5$
2	24	$14 \cdot 10^5$	$6 \cdot 10^5$	$7 \cdot 10^5$
5	3	$14 \cdot 10^5$	$8 \cdot 10^4$	$1 \cdot 10^5$
5	6	$14 \cdot 10^5$	$3 \cdot 10^3$	$2 \cdot 10^3$

C. stellatoidea was incubated in broth medium at 30 C; after indicated exposure times, samples were plated onto growth medium agar with or without 200 µg cysteine per milliliter. Colonies were counted after 72 hr incubation.

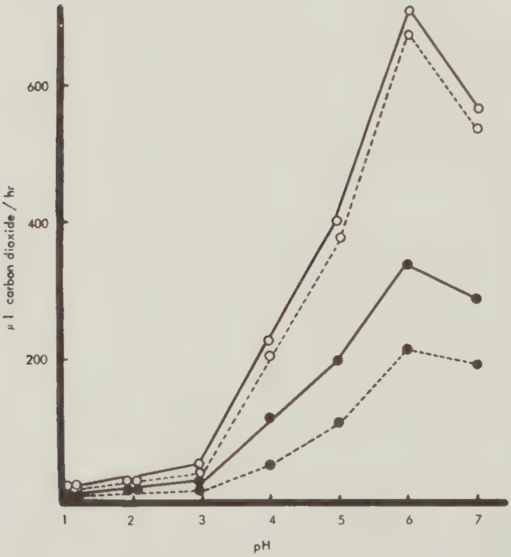

Fig. 2. Effect of sodium and potassium ions on glucose fermentation in acetate buffer. Warburg vessels contained 5 mg dry weight Candida stellatoidea, 100 mg glucose, appropriate mixtures of HCl, acetate acid, and sodium acetate (●) or potassium acetate (O), with (dotted lines) or without (solid lines) 4 µg nystatin per milliliter, and a nitrogen atmosphere.

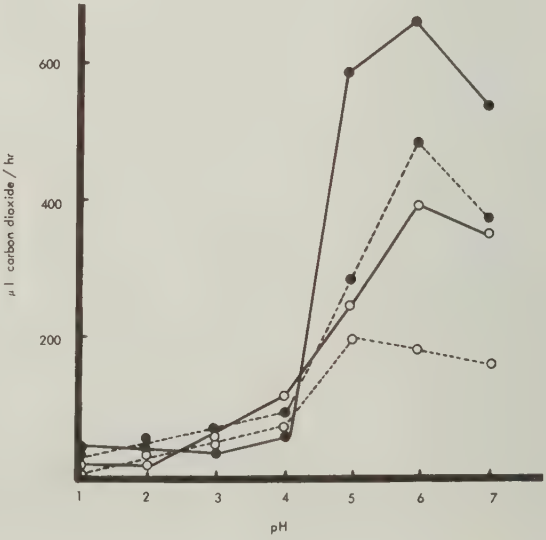

Fig. 3. Effect of pH on glycolysis by the nystatin resistant variant (●) and the sensitive parental strain (O) in 0.067 M sodium acetate with (dotted lines) or without (solid lines) 4µg nysatin per milliter.

Table 5. Effect of Polyenes on Glycolysis by Candida stellatoidea

Polyene	Carbon dioxide evolved, μl/hr		
	0 μg/ml	5 μg/ml	25 μg/ml
Amphotericin A	520	510	280
Amphotericin B	520	585	275
Endomycin	520	660	540 (50 μg = 445)
Filipin	520	500	80
Nystatin	520	170	20
Pimaricin	520	510	350

Warburg vessels contained 5 mg dry weight washed yeast, pH 4.5, 0.067 M NaH_2PO_4, 100 mg glucose, and a nitrogen atmosphere.

of our study we became concerned about the relationship between growing cells in nutritive medium and washed cells in buffer plus sugar or other carbon source. Candida in the exponential phase of growth, along with its growth medium, was dispensed into Warburg vessels and the effects of minimal growth-inhibiting levels of polyenes on respiration and fermentation were measured. It should be noted that by the exponential phase the yeasts had lowered the pH of the broth to 3, which was the acidity wherein polyenes were most powerful. As little as 1.5 μg nystatin per milliliter halted glucose utilization completely. Cultures adjusted to pH 4.5 before addition of drug were not retarded with respect to glucose metabolism but were unable to grow significantly (Table 7). Cell-free preparations of the parental and mutant strains were made by shaking a yeast paste with glass beads for 150 min at 4 C [11], and the action of nystatin upon the opalescent suspensions, from which whole cells and cellular debris had been removed by centrifugation, was assayed manometrically. At concentrations of 4 and 10 μg/ml the drug stimulated anaerobic production of carbon dioxide from glucose by cell-free preparations, whereas intact cells of the wild type and of the resistant strain were inhibited substantially by the higher level of antibiotic (Table 8). The effects of the enzyme poisons, sodium iodoacetate and sodium fluoride, on glycolysis by intact cells were determined. The assays were carried out in four different salt solutions, each used at the optimal hydrogen ion concentration for glucose fermentation. Both the antibiotic-resistant and the sensitive strains were inhibited by 10^{-4} M iodoacetate when the suspending 0.067 M acid—

Table 6. Effect of Nystatin on Oxygen Uptake

Substrate	Oxygen consumed, μl/hr	
	Control	2 μg Nystatin /ml
Acetate	16	19
Citrate	26	147
Gluconate	74	128
Glutamate	190	201
Lactate	131	123
Succinate	188	173
Glucose	244	210

Warburg vessels contained 5 mg dry weight washed yeast, pH 4.5, 0.067 M NaH_2PO_4, 100 mg carbon source, and an air atmosphere. Respiratory CO_2 was removed with 20% potassium hydroxide.

Table 7. Effect of Polyenes on Respiration and Fermentation by Growing Cells

Polyene	μg/ml	Gas measured	pH of medium	Gas volume, μl/hr
Amphotericin A	1.0	CO_2	4.5	490
	1.0	O_2	4.5	210
Amphotericin B	1.0	CO_2	4.5	475
	1.0	O_2	4.5	202
Endomycin	5.0	CO_2	4.5	470
	5.0	O_2	4.5	200
Filipin	2.0	CO_2	4.5	475
	2.0	O_2	4.5	206
Nystatin	1.5	O_2	3.0	13
	1.0	O_2	4.5	235
	1.5	CO_2	3.0	25
	1.0	CO_2	4.5	490
Pimaricin	5.0	CO_2	4.5	460
	5.0	O_2	4.5	225
None	0	O_2	3.0	170
	0	O_2	4.5	216
	0	CO_2	3.0	315
	0	CO_2	4.5	465

Warburg vessels contained 3 ml of 12-hr culture of C. stellatoidea in growth medium ($1.7 \cdot 10^8$ cells per milliliter). Carbon dioxide evolution was measured in a nitrogen atmosphere; oxygen uptake was measured in air. Respiratory carbon dioxide was removed by potassium hydroxide.

salt solution was pH 2 sodium borate or potassium phosphate, but neither organism was inhibited in pH 7 sodium borate, pH 5 potassium phosphate, pH 5 potassium phthalate, or pH 6 sodium acetate. Also, the wild type and mutant were unable to ferment glucose in the presence of 10^{-2} M sodium fluoride if the suspending medium was pH 2 borate, pH 5 phthalate, or phosphate at pH 2 or 5; only in pH 6 acetate or borate at pH 7 was sodium fluoride harmless (Table 9). Dinitrophenol (10-25 μg/ml) in 0.067 M sodium phosphate at pH 5 stimulated oxygen uptake when glucose was the substrate. Mixtures of stimulatory levels of this poison and subinhibitory amounts of nystatin reduced oxygen consumption greatly. Repressive concentrations of nystatin, whether in combination with dinitrophenol or not, decreased respiratory activity (Table 10).

Table 8. Effect of Nystatin on Glucose Fermentation by Cell-Free Preparations

Strain	Preparation	Carbon dioxide evolved, μl/hr		
		0 μg Nystatin/ml	4 μg Nystatin/ml	10 μg Nystatin/ml
CS	Whole cells	616	735	305
	Cell-free	935	1100	1390
CR	Whole cells	620	590	411
	Cell-free	442	565	620

Growth of strain CS was inhibited by 1 μg nystatin per milliliter, whereas strain CR grew in the presence of 20 μg/ml. Cells were disrupted by shaking with glass beads for 150 min at 4 C. Whole cells and cell debris were removed by centrifugation. Carbon dioxide evolution was measured manometrically.

Table 9. Effect of Iodoacetate and Fluoride on Glycolysis

Buffer	pH	Strain	Carbon dioxide evolved, μl/hr		
			Control	Iodoacetate	Fluoride
Acetate	6	CS	390	360	310
		CR	685	655	645
Borate	2	CS	90	27	2
		CR	155	0	0
	7	CS	85	81	87
		CR	155	150	155
Phthalate	5	CS	390	360	10
		CR	625	625	20
Phosphate	2	CS	405	69	10
		CR	731	83	35
	5	CS	444	400	10
		CR	705	695	27

The nystatin-sensitive strain (CS) and resistant strain (CR) were suspended in 0.067 M potassium salt solution at the indicated pH. Carbon dioxide produced from 100 mg glucose by 5 mg dry weight of washed cells was measured manometrically. When indicated, sodium fluoride and sodium iodoacetate was added to a final concentration of 10^{-2} M and 10^{-4} M, respectively.

Finally, the nystatin-resistant variant and the sensitive parental strain were compared in growth studies. Inhibitors used included the polyene antibiotics nystatin, filipin, endomycin, amphotericin A, amphotericin B, and pimaricin, the chemically unrelated antifungal antibiotics griseofulvin and cycloheximide,

Table 10. Interactions between Nystatin and 2,4-Dinitrophenol

Nystatin, μg/ml	Dinitrophenol, μg/ml	Oxygen consumed, μl/hr
0	0	247
1	0	312
2	0	234
5	0	194
10	0	19
0	10	242
0	25	255
0	50	150
1	10	266
1	25	93
1	50	40
2	10	117
2	25	106
2	50	60
5	25	44
10	50	0

Warburg vessels contained 5 mg dry weight washed yeast, pH 4.5, 0.067 M NaH PO_4, 100 mg glucose and an air atmosphere. Respiratory CO_2 was removed with 20% potassium hydroxide.

and the metabolic poisons sodium iodoacetate and 2,4-dinitrophenol. The latter two groups were equally potent in preventing growth of the mutant and wild type Candida. All of the polyenes reduced cellular proliferation of the parental strain more than the nystatin-resistant strain. Cross-resistance was least evident, but measurable, with pimaricin.

Table 11. Susceptibility of a Nystatin-Resistant Strain to Polyenes and Other Inhibitors

Inhibitor	Minimal inhibiting concentration, μg/ml	
	CS	CR
Amphotericin A	1	50
Amphotericin B	1	25
Cycloheximide	50	50
2,4-Dinitrophenol	50	50
Endomycin	5	25
Filipin	1	50
Griseofulvin	50	50
Iodoacetate	0.2	0.2
Nystatin	1	22
Pimaricin	5	10

Sterile antibiotic or enzyme inhibitor was added to cool, molten growth medium agar; individual plates were seeded with approximately 10^4 cells of the nystatin-sensitive strain (CS) or the resistant strain (CR) and incubated at 30 C for 4 days.

DISCUSSION

Although it was possible to contrive conditions where the population of viable cells in low concentrations of polyenes remained constant for several hours, the primary effect of these drugs was fungicidal. It should be noted that the power of these antibiotics was influenced markedly by hydrogen ion concentration [12, 13], cation content [14, 15], and anion content [10] of the medium. These complications became a serious problem in studies on the mode of action of polyenes. The primary, vulnerable, physiological locus may well be different in dissimilar environs. What should be the bases for selecting conditions for such research? More nystatin is bound in acid milieu than in neutral or alkaline milieu [16] and fungicidal activity and suppression of glycolysis are greatest at pH 2, which is the pH of medium during late exponential and stationary growth phases. Therefore, should work on mechanisms of action be conducted in a markedly acid environment? On the other hand, C. stellatoidea is a saprophyte in the normal, adult vagina, which is pH 5; moreover, the optimal pH for glycolysis and growth initiation is 4.5-5. From a clinical point of view polyenes must act in saliva, intestinal contents, blood, and tissue fluids, all of which are maintained near neutrality. Should mode of action studies aim to duplicate optimal growth conditions or human-physiological conditions? These are not the only difficult questions facing the student of drug function. On what basis does an investigator decide which of several changes in the sensitive microbe is the critical site? It is tempting to direct attention to gross alterations and to those easily measured. Minute changes in a reaction rate or slight disorganization of internal cellular integration is not easily detected. Alas, drug action may involve two or more simultaneous events, sequential events, or both. Nevertheless certain premises for mode of action studies can be established. The drug concentrations used should correspond to minimal inhibiting doses according to in vivo or in vitro tests. The effects considered significant should occur prior to or concurrent with suppression of growth. The test conditions should be related to "natural" conditions. Too many experiments, including some of ours, have employed excessive drug levels, cells long since dead, and environments in which the assay organism could not function as a biological entity.

Although interpretations of the action mechanisms of polyenes are diverse, the results reported by various workers using different antibiotics and different indicator strains have been remarkably similar. In standard manometric experiments polyenes at minimal growth-inhibiting concentrations stimulated oxygen uptake and respiratory or fermentative production of carbon dioxide [17, 18]. On this basis it has been proposed that polyenes act like 2,4-dinitrophenol [19]. We do not accept the hypothesis that the mechanism of nystatin involves uncoupling of phosphorylation because low levels of drug do not prevent induced enzyme synthesis whereas dinitrophenol does [9]. Incidentally, this also indicates that ribonucleic acid synthesis is not the primary site of nystatin action. Moreover, growth of the nystatin-resistant strain is as susceptible to dinitrophenol as that of the parental strain and much more antibiotic is required to affect glycolysis or respiration, at least above pH 4.5, than to prevent cellular proliferation. Additionally, Candida cannot grow in medium containing α-ketoglutarate or glutamate as the carbon source plus low levels of nystatin even though utilization of these substances is not impaired by drug concentrations which halt oxidative or fermentative degradation of glucose [20]. It must be admitted that there are some unexplained interactions between nystatin and dinitrophenol. The severe suppression of respiration by mixtures of noninhibitory levels of dinitrophenol and nystatin may result from increased penetration of the uncoupling agent and the loss of orthophosphate through the antibiotic-damaged membrane [21-23]. This view is consistent with the observation that citrate, which is not able to enter normal Candida cells unless they are washed severely, is degraded by nystatin-treated yeasts. This finding supports the opinion that selective permeability is altered by polyenes.

The ability of cysteine and reducing compounds to protect against polyenes suggests that these drugs act on sulfhydryl groups which are abundant in the cellular membrane. The failure of cysteine to save nystatin-damaged cells, however, is indicative of an interaction with the polyene rather than with cellular components. Similarly, the ability of cholesterol or ergosterol to protect fungi from the deleterious action of polyenes can be attributed to formation of an inactive sterol-antibiotic complex [24, 25]. Another indication of an effect on permeability is the differential susceptibility of glycolysis to nystatin, depending upon the sodium or potassium ion content of the medium. Potassium ions protect glycolysis but not growth. Another indictment of permeation as the vulnerable site is that cell-free preparations can ferment glucose in the presence of antibiotic levels that inhibit intact cells [26]. Interference with respiration and fermentation by polyenes may be the result of blocking permeation of glucose or loss of cofactors for these reactions. The ready entrance of citrate into damaged cells and the rapid loss of phosphate are consistent with an over-all effect on selective permeability. These actions are also observed with detergents. In fact, almost all of the findings with polyenes parallel those with surface-active agents: pH-dependency, protection by cations and nucleic acid, binding to cell exterior, stimulation of respiration and glycolysis by low levels and suppression by high levels, and leakage of phosphate-containing material [27]. Moreover, resistance is rare and stepwise. It should be noted though that uranyl ions, which are very effective in protecting against polymyxin, do not lessen nystatin toxicity. On the other hand, Tween 80 promotes growth of mycobacteria; similarly we have found that nystatin promotes dispersed growth of the related microbe *Nocardia farcinica*.

In formulating a hypothesis concerning mode of action of polyenes, the insensitivity of metabolically inactive cells must be considered. Yeasts kept at 4 C or starved cells in buffer are not killed by fungicidal concentrations of drug. Also, polyenes may be used to select for growth-factor-dependent mutants, much as penicillin is used for bacteria [28]. To account for this, we suggest that polyenes are surface-active agents which are incorporated specifically into the cellular membrane by an energy-requiring reaction.

SUMMARY

The polyene antifungal antibiotics amphotericin A, amphotericin B, endomycin, filipin, nystatin, and pimaricin inhibited respiration and fermentation by *Candida stellatoidea* at concentrations five- to tenfold higher than the minimal levels that prevented growth. Growth suppression caused by amphotericin B, filipin, nystatin, and pimaricin was negated by 100-500 μg/ml cysteine or ascorbate. Retardation of glycolysis by nystatin was greater in 0.067 M sodium acetate than in potassium acetate. Anaerobic degradation of glucose by a mutant, which grew in the presence of twentyfold more nystatin than the parental strain, was inhibited by the same drug concentrations that impaired metabolic activity of the latter organism. The resistant variant produced twice the volume of carbon dioxide per unit mass as the wild type strain. Cell-free preparations of the sensitive and the resistant strains were able to ferment glucose at antibiotic levels which interfered with intact cells. A mutant selected for resistance to nystatin possessed increased resistance to other polyenes. The mechanisms of action of these antibiotics seem to be similar to one another and to those characteristic of surface-active agents.

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FACTORS LEADING TO OVERT MONILIAL DISEASE

II. Retardation of Growth of Candida albicans by Metabolic End Products of Intestinal Bacteria

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INTRODUCTION

Data presented in an earlier report from this laboratory [1] permitted the classification of the usual bacterial residents of the human gastrointestinal tract as stimulatory, indifferent, and inhibitory with respect to the growth of *Candida albicans* in vitro. It was demonstrated that none of the inhibitory bacteria produced anti-candidal fungicides and the suggestion was made that the bacterial inhibition of yeast growth was due to metabolic end products of these organisms. This report is concerned with the evaluation of certain bacterial metabolic end products on the growth of *C. albicans*. Since the original investigation was made with the yeast and bacteria separated by a dialysis tube in Eugon broth (BBL) only low molecular weight materials that could be elaborated by bacteria in the medium used were studied [2–5]. In addition, related compounds were studied, which were not necessarily of bacterial origin but had features which appeared to enhance inhibition of the yeast growth.

MATERIALS AND METHODS

Most of the materials and methods were described earlier [1]. Thus, *C. albicans* No. 1539 was the test organism. Eugon broth (BBL) served as medium. Cell yields were determined by optical density measurement at 650 mμ, within the limits established before.

The compounds to be tested were dissolved in Eugon broth, sterilized by Seitz filtration, and dispensed into sterile screw cap tubes. Autoclaved Eugon

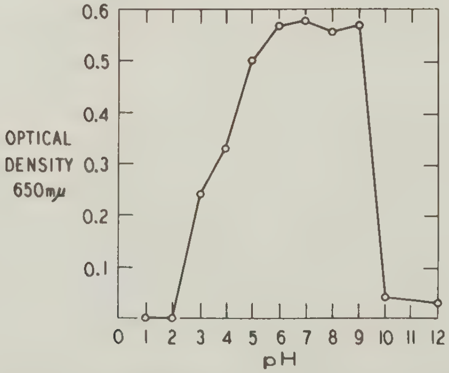

Fig. 1. Effect of pH on the growth of *Candida albicans* in Eugon broth.

broth was then added to a final volume of 9.0 ml. All experiments were set up in quintuplicate. Eugon broth without any addition accompanied each experiment. All tubes were inoculated with 0.05 ml of a 24-hr culture of the yeast. The OD of the yeast growing within the control tubes were used as the indicator of 100% growth.

The temperature of incubation was 37 C. Atmospheric conditions were aerobic since this atmosphere favored the detection of inhibitory end products.

While C. albicans grew well in Eugon broth over a comparatively wide pH range (Fig. 1), initial pH was always maintained at neutrality since the penetrability of organic acids is enhanced in the acid range [6].

RESULTS

The data in Table 1 illustrate that in concentrations which might be anticipated from the glucose content of Eugon broth (0.027 M), many of the most commonly encountered metabolic end-products of the intestinal microflora exert little effect on the yeast. Of the substances tested only butyric acid and, to a lesser degree, succinic acid hinder the proliferation of the yeast. C. albicans responded to all of the compounds listed in higher concentrations but it is unlikely that such accumulations could occur under natural conditions. Therefore, only butyric acid and succinic acid are regarded as possible inhibitors of the yeast.

Table 1. The Effect of Bacterial Decomposition Products of Glucose on the Growth of Candida albicans

Substance	Normal concentration* of metabolite	Strength of metabolite		
		0.01x	1x	100x
Formic acid	0.014 M	94.5†	73†	9†
Acetic acid	0.012 M	100	76	8
Ethanol	0.019 M	88	83	2.5
Lactic acid	0.084 M	86	81	3.7
Succinic acid	0.008 M	62	62	12
Butyric acid	0.075 M	104	34	3.3
Butanol	0.016 M	104	85	2.3
Acetone	0.013 M	113	113	2.5
Isopropanol	0.013 M	96	92	3.2
2,3 Butylene glycol	0.017 M	100	100	3.8

*Computed on the basis of 0.027 M glucose present in Eugon broth (BBL).

†Percent growth

A variety of other bacterial end-products or metabolic intermediates likely to accumulate in the medium were tested for their anti-candidal properties in concentrations ranging from 0.001 M to 0.05 M (Table 2). Only slight inhibition of the yeast was achieved in the lowest concentrations. Diacetyl curtailed Candida when present in 0.01 M amounts while the same concentrations of indole and suberic acid resulted in roughly 50% reduction of cell yields. A number of the compounds showed no effect when present in 0.05 M solution but this concentration of 1,3-propanediol, α-ketoglutaric acid, n-amyl alcohol, acetoin, glutaric acid, adipic acid, and pimelic acid inhibited Candida.

These findings led to the investigation of certain related compounds. Figure 2 represents the results when monocarboxylic straight-chain fatty acids are

Table 2. The Response of Candida albicans to Some End Products of Bacterial Metabolism

Substance	Growth, %		
	0.001 M	0.01 M	0.05 M
Indole acetic acid	117	85	ppt*
Indole propionic acid†	117	87	ppt*
Indole†	49	54	ppt*
α-Ketoglutaric acid	79	76	28
1,2 Propanediol	105	110	106
1,3 Propanediol	94	94	8.5
Glycerol	102	105	100
Diacetyl	87	4.5	5
Acetoin	96	92	27
Fumaric acid	100	100	100
Urocanic acid	100	100	98
Glutaric acid	80	78	55
Adipic acid	99	94	56
Pimelic acid	100	91	48
Suberic acid	98	60	–
Sebacic acid	94	90	–
2-Butanol	100	97	87
3-Butanol	99	97	100
n-Amyl alcohol	97	94	18

*Precipitate formed in this concentration.

†Dissolved in minimal amounts of ethanol, not inhibitory to the yeast.

introduced into the medium. While the solubility of some of these compounds did not permit the evaluation in all the various molar concentrations, the aliphatic acids with chain lengths of 6 to 9 carbons were quite inhibitory in comparatively low concentrations, confirming similar observations for other yeasts [7, 8] and

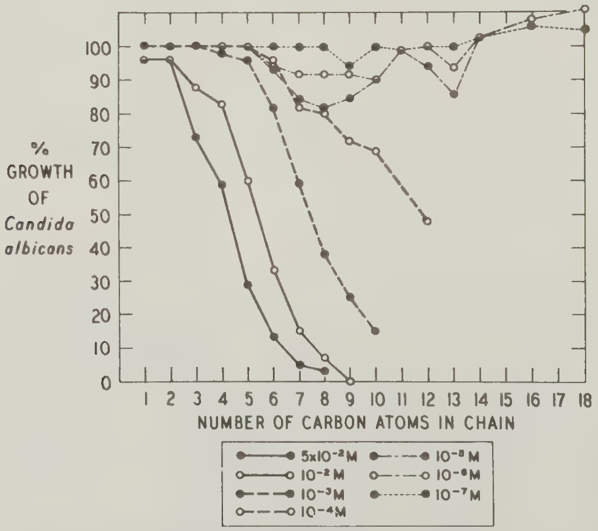

Fig. 2. Response of Candida albicans to monocarboxylic n-fatty acids (K salts) at pH 7.0.

for fungi [9]. The inhibitory nature of these acids is illustrated especially by the curves representing the lower concentrations. These display a slight but definite depression of cell yields in the region of these acids.

Figure 3 summarizes the influence of the decarboxylated amino acids. The concentrations used again delineate the most active inhibitors. Thus phenylalamine, valamine, and prolamine are quite active at the 0.01 M level and their activity increases with an increase in the amount present. Other decarboxylated compounds such as ethanolamine or histamine are either completely inactive or display limited activity only in high concentration. The experiments with the fatty acids and amines led to the study of heptylamine and octylamine, compounds which are not normal end-products of bacterial metabolism. Nevertheless, when present in small amounts, their rather excellent anti-candidal properties (Fig. 3), substantiate the observation that the aliphatic compounds with intermediate length chains are inhibitory to Candida and that amino groups may be substituted for the carboxyl moiety.

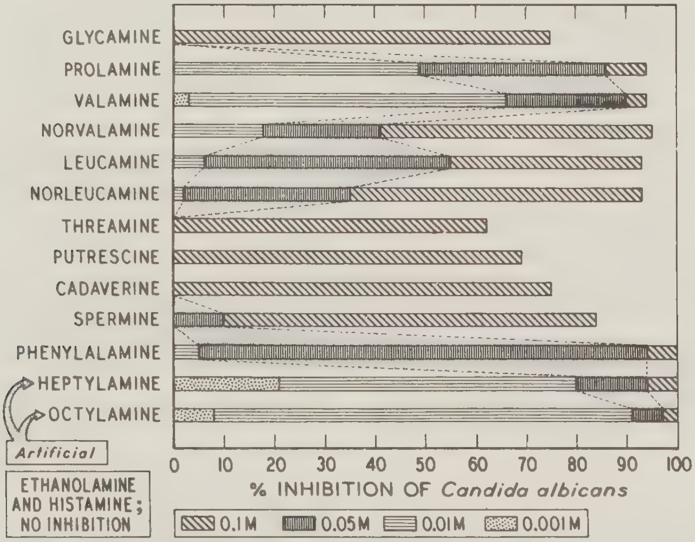

Fig. 3. Inhibition of *Candida albicans* by decarboxylated amino acids.

The detrimental effect of hydrogen sulfide was studied with the gas as well as with the sodium salt. Findings suggested inhibition when the gas was used, but evaluation was difficult because of precipitates formed. Sodium sulfide did not inhibit the yeast proliferations in concentrations ranging from 10^{-7} M to 10^{-3} M. With more concentrated solutions, precipitates again obscured analysis. This problem requires further study. Other end-products of bacterial metabolism, including vitamins, purines, pyrimidines, and their intermediates, did not display inhibitory capacities even in high concentrations.

DISCUSSION

It was hoped that this study of cell-free metabolic end-products would detect inhibitory as well as stimulatory compounds. Only the data concerned with compounds of an in vitro inhibitory character with respect to *C. albicans* were presented here. Those with stimulatory capacities are still under investigation.

The findings do suggest that certain compounds, derived from bacteria with which C. albicans may establish itself as a coinhabitant of the human gastrointestinal tract, may be involved in controlling the yeast. There is a clear-cut ability of these organisms classified as inhibitory to produce some of these compounds. Thus, diacetyl has been postulated as an end-product of Klebsiella-Aerobacter metabolism resulting from the oxidation of acetoin. This organism ranked high among those classified as inhibitory. While experimental difficulties prevented the confirmation of Bergeim's findings with sulfide [8], the inhibitory activities of Escherichia freundii and Proteus mirabilis may also be explained by the production of metabolites which would even enhance the precipitating effect of the sulfide. The action of organic acids can certainly be correlated with the activities of most of the anaerobic bacteria, especially the clostridia, while the amines suggest a link with a number of the members of the family Enterobacteriaceae [10]. It is interesting that the corresponding dicarboxylic acids or alcohols did not manifest the same degree of inhibition.

It should be pointed out that a number of the compounds displaying inhibition could also be elaborated by organisms which are among those which we have classified as stimulatory or indifferent. Indeed, indole produced by the "indifferent" Escherichia coli does inhibit the yeast in 0.01 molar solution. It is, however, unlikely that such a concentration is achieved in nature or even with organisms growing in an unsupplemented medium.

While these observations may have little application in the control of disease produced by Candida, bacterial metabolism may influence the yeast on an ecological basis. The retardation of growth of a few yeast cells in an environment populated by bacteria may be due to the elaboration by certain bacteria of compounds which, in extremely small quantities, prevent multiplication of the yeast. If such curtailing bacteria are removed from the vicinity of Candida, by chance or by interference in the form of antibiotics, competing bacteria, or host defenses, the yeast may then begin to grow unopposed and establish itself as a coinhabitant. These in vitro relationships between Candida, bacteria, host factors, and antibiotics are now under investigation.

CONCLUSIONS

The anti-candidal properties of low molecular weight end-products of bacterial metabolism were investigated. Certain fatty acids, amines, and diacetyl were found effective against the yeast. Simple synthetic compounds, heptylamine and octylamine, were similarly active against Candida albicans.

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DISCUSSION

DR. PETERSON: I'd just like a little information on these last two papers. Dr. Bradley mentioned that many of these materials complex nystatin and I was wondering if the last author could say whether or not there are enough of these complexing materials in the intestinal tract under certain diets and not in others to explain some of the conflicting results we have had concerning the inhibition of diarrhea you get with these drugs.

DR. ISENBERG: I hope I understand the question correctly. We have not worked with stools as such; we worked with the representative microorganisms of the normal microflora of the gastrointestinal tract and have used them in culture. I could not answer your question with regard to diarrhea on this basis.

DR. PETERSON: My point is that perhaps these conflicting results are not as conflicting as they seem. They just have not completed the experiments, if dietary or other areas could explain it.

NONMEDICAL USES OF ANTIBIOTICS IN GREAT BRITAIN

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INTRODUCTION

In the United States, a scant eight years after the first observation of growth stimulation by antibiotics [1], a half million pounds of antibiotics were produced annually for feed supplement alone [2]. There followed shortly the use of antibiotics in food preservation (poultry and fish) and also the application of antibiotics in plant disease control.

Since all of these nonmedical uses relate directly or indirectly to food consumption by humans, considerable interest exists with regard to potential public health problems and, in fact, changes in the food additives regulations of the United States have been stimulated [3].

This would seem, therefore, a propitious occasion to examine the status of nonmedical applications of antibiotics in Great Britain from whence the first major antibiotic was given to the world.

CONTROL AND REGULATION

Primarily two ministries (acting separately or jointly) exercise control over antibiotics in Great Britain. They are the Ministry of Health and the Ministry of Agriculture, Fisheries, and Food. The ministries are guided by committees specifically appointed to study and evaluate data and evidence and to make recommendations with regard to petitions for changes and additions to existing law. Thus the Food Standards Committee of the Ministry of Agriculture, Fisheries, and Food is concerned primarily with preservatives in food. An Antibiotics Panel was appointed by them in 1956, consisting of agricultural and medical experts, to advise this committee on use of antibiotics as food preservatives, and from this evaluation legislative recommendations are being made. The panel was specifically responsible for "advising on the extent of the health hazard involved in the use of antibiotics prepared for the preservation of food and for use in agriculture" [4].

ANTIBIOTICS IN ANIMAL FEEDS

The first statutory regulation on nonmedical use of antibiotics in Great Britain came in 1953. It originated from the Ministry of Health and was a modification of the Penicillin Act of 1947. It specifically exempted Aureomycin (chlortetracycline) and penicillin in feeding-stuffs from "the control of sale and supply" applying to therapeutic forms of these compounds [5]. A proviso in this act limited the maximum concentration of these antibiotics in feeds to 100 ppm. In 1955 [6], oxytetracycline was added to penicillin and chlortetracycline to make a total of three antibiotics permitted in Great Britain as feed supplements, which were limited to use in pigs and poultry.

Table 1 summarizes the regulatory status of antibiotic feeds for most of the countries of Europe. It illustrates permissible antibiotics, maximum levels, and animals which may be fed.

Table 1. European Regulations for Antibiotic Feed Supplements

Country	Antibiotics	Animals	Maximum level, ppm
Austria	OTC-CTC-Penicillin	Pigs-poultry	60
Belgium	OTC-CTC-Penicillin-Bacitracin	Calves-pigs-poultry	50
Denmark	OTC-CTC-Penicillin	Young growing animals	25
Finland	OTC-CTC	Pigs-poultry-fur bearers	50
France	OTC-CTC-Penicillin-Bacitracin*	Pigs-poultry	200
Germany	OTC-CTC-Penicillin	Pigs-poultry-calves	200
Great Britain	OTC-CTC-Penicillin	Growing pigs-poultry	100
Holland	Not specified	Pigs-poultry-calves	100
Norway	OTC-CTC-Penicillin	Pigs-poultry-calves	50
Sweden†	OTC-CTC-Penicillin	Pigs-poultry-calves-mink	20
Switzerland	OTC-CTC-Penicillin-Bacitracin	All except dairy cattle	50

Ireland, Greece, Israel, Italy, and Portugal have no restrictions

*Also seven other antibiotics
†Anticipated regulations

It should be noted here that in Great Britain promulgation of feed supplement regulations did not go into effect without some concern, particularly regarding the emergence of antibiotic resistant strains of bacteria in animals ingesting these feeds. This was based on the fact that much literature had accumulated in the United States and Great Britain from the medical fields, indicating the wide scope and complexity of antibiotic resistance [7-9].

Nevertheless, published experimental data indicating emergence of antibiotic-resistant bacteria from dietary antibiotics was lacking prior to the date of these regulations. This was not only true in Great Britain but also in the United States, where antibiotics were approved by the Food and Drug Administration in 1951 [10]. Thus, the literature was replete with examples of the growth stimulating effect of antibiotics but little or nothing was available on the emergence of antibiotic-resistant flora. However, beginning in 1957 and subsequently, there began to appear in the British literature reports of high incidence of resistant enteric flora following use of antibiotic feed supplement.

Clear-cut evidence appeared that such organisms as Escherichia coli, faecal streptococci, Staphylococcus aureus, and Salmonella typhimurium would show high incidence of resistance following dietary antibiotics.

Smith and Crabb [11] and Barr and Carman [12] showed that poultry fecal samples contained extremely high percentages of tetracycline resistant E. coli following use of tetracycline in feed supplement. In the latter instance fatal diarrhea developed in broiler chicks due to tetracycline resistant E. coli. Barnes [13] showed faecal steptococci developed antibiotic resistance on poultry feed at varying levels of CTC (chlortetracycline).

Similarly, Gordon et al. [14] showed that when CTC was fed at 25-400 ppm it would elicit increased resistance of E. coli in chicks. Indeed, up and down fluctuations of resistant coliforms could be induced by removal and addition of the CTC.

More recently Smith and Crabb [15] have shown that tetracycline and penicillin feeding has increased the tolerance of antibiotic-resistant S. aureus in chickens and swine. In addition human attendants working in areas where antibiotic supplement was used had a greater percentage of resistant S. aureus than those working where these supplement were not used.

One of the few studies on pathogens in this area has been that of Garside et al., 1960 [16]. They found that S. typhimurium would increase its resistance to CTC tenfold if infected chicks were on CTC diet. Since many feed stuffs are frequently contaminated with Salmonella, [17] and poultry constitute one of the main sources of Salmonella [16], emergence of CTC-resistant Salmonella was considered a very real possibility in a natural situation. However, it should be noted that tetracyclines are not normally the antibiotic of choice for Salmonella infection and therefore the significance of this phenomenon with regard to infection in man and animals remains uncertain.

There is also evidence that, in addition to change in antibiotic resistance pattern, there is possible a change in the serological type of the organism. Thus, Elliott and Barnes [18] proved that dietary CTC influenced the serologic type of faecal streptococci which predominated in the gut of poultry. CTC feeding led to predominance of CTC resistant Streptococcus faecalis (H69D5) withdrawal resulted in reappearance of original types D15 and D76 with lower antibiotic resistance.

This phenomenon is now being studied at the Poultry Research Station, Houghton, with regard to emergence of pathogenic, antibiotic-resistant E. coli from poultry flocks, with a view of learning the details of E. coli septicemia observed in poultry on antibiotic feeds.

At the current moment there is underway in Great Britain a reinvestigation of the entire field of antibiotics in animal feeds. This has been prompted by the industry's desire to expand this use of antibiotics by requesting the regulations be extended to include breeding and laying stocks in addition to animals destined for slaughter. On the other hand, the recent publications indicating emergence of antibiotic resistance have concerned the public health authorities. As a result, a committee consisting of Agriculture Research Council and Medical Research Council members is carefully examining the position with regard to antibiotics in feed stuffs.

ANTIBIOTICS AS FOOD PRESERVATIVES IN GREAT BRITAIN

Three antibiotics, chlortetracycline, oxytetracycline, and nisin,were recently considered by the Food Standards Committee of the Ministry of Agriculture, Fisheries, and Food for use as food preservatives in Great Britain, and the aforementioned Antibiotics Panel considered the extent of the health hazard involved. Their report [4] discusses the problems involved, giving reasons for either the rejection or acceptance of each proposed usage. It is interesting to note that the panel considered evidence from industrial companies, published literature, the United States Food and Drug Administration, and information supplied from the Low Temperature Research Station, Cambridge, England and the Torry Research Station, Aberdeen, Scotland. The latter two British Government laboratories provided specific answers to questions the panel considers most significant. Following publication of this report a deadline was set of March 25, 1961 for criticisms and objections. When these have all been considered by the Food Standards Committee, amendments to the Public Health Regulations (Preservatives, etc. in Food) will be introduced.

Chlortetracycline and oxytetracycline. One of the main problems concerning chlortetracycline and oxytetracycline as food preservatives relates to the influence such a use may have on their effectiveness as therapeutic agents. Three treatments considered by the Antibiotics Panel were for poultry, fish, and whales. Points of interest specifically considered were degradation on cooking, active residues, development of resistance, hypersensitivity, and standards of hygiene.

It had been proposed that eviscerated poultry should be cooled in ice slush containing 10-20 ppm antibiotics as is the practice in some poultry plants in the United States. The panel was satisfied that the residues in poultry would be insignificant after cooking but they were concerned that under certain conditions there might be a danger from the multiplication of resistant food poisoning organisms (in particular salmonellae) on the treated carcasses, as has been shown by Hobbs et al. [19]. This problem relates to the fact that only about 19% of all British households have refrigerators and the antibiotic-treated poultry may be stored by the housewife for 1-2 days at normal temperatures before cooking.

Investigations are still being carried out by the Torry Research Station, Aberdeen, on the residual antibiotic in cooked fish (cod, haddock, plaice, and sole). Although most of the antibiotic is destroyed in the fish muscle, significant residues have been found in the skin. Similar residues in skin and slime have also been reported in Japan by Tomiyama [20]. The Antibiotics Panel have therefore reserved their decision on this application pending further information.

It has now been shown in a number of trials with whale products that the introduction of chlortetracycline or oxytetracycline to give an over-all level of about 1 ppm may delay the onset of deep spoilage considerably and, hence, increase the quality of products. It is not yet clear whether administration through the harpoon head or through the pipe line used to inflate the carcass is the most efficient method. The Food Standards Committee was only concerned with those products used in Great Britain for human consumption and concluded that whale meat extract and whale oil products derived from tetracycline treated whales did not present a hazard if the processing was of a nature by which all the antibiotic was destroyed.

Nisin. The antibiotic nisin is produced by the growth of certain strains of the cheese starter organism Streptococcus lactis. Following the work of Whitehead [21] it was eventually isolated and characterized by Mattick and Hirsch [22,23]. It was originally developed for therapeutic use but, after careful investigation, Gowans et al. [24] concluded that it was of no medical value especially as adequate therapeutic levels of nisin are soluble only in aqueous solutions at pH 3.0 or lower and oral administration was precluded because of its destruction by trypsin.

Crystalline nisin is a large polypeptide or small protein with a molecular weight of approximately 10,000 and having an activity of about 40,000,000 Reading units/g. The solubility of nisin is relatively high in acid solution and decreases rapidly towards pH 7.0. It has a very low toxicity and its destruction by trypsin precludes any risk of nisin altering bacterial flora of the gut. It has no activity against most Gram-negative organisms but has been shown to be inhibitory to various species and strains of the genera Staphylococcus, Streptococcus, Bacillus, Clostridium, and Corynebacterium. The in vitro activity of nisin against a number of organisms concerned in food processing and food spoilage is given in Table 2. Nisin-resistant bacteria are not cross-resistant to the medical antibiotics nor to the other polypeptide antibiotics such as subtilin, bacitracin, polymyxin, circulin, micrococcin, and diplococcin [25].

A great deal of the spoilage which occurs in processed cheese due to clostridial growth can be controlled by nisin [26]. A method was developed in Great Britain for producing a cheese containing 15,000 nisin units/g which could be processed together with the ordinary cheese and so inhibit the Clostridia [27]. It has been found that nisin occurs naturally in a number of cheeses which are not liable to clostridial spoilage whilst certain other cheeses such as Gruyere, Emmental, Semsoe, Edam, Gouda, and Parmesan are very often spoiled by these organisms. The Food Standards Committee decided that there was no hazard

Table 2a. Antimicrobial Spectrum of Nisin Against Clostridium

Species	Number of strains tested	Sensitivity, units/ml
C. bifermentans	2	50-200
C. bifermentans	1	1.5
C. botulinum A (1) and B (1)	2	100
C. botulinum C (1), D (1), E (1)	3	25-50
C. multifermentans	2	25-50
C. multifermentans	1	>200
C. parasporogenes	2	25-30
C. putrificum	1	15
C. putrificum	3	100-200
C. sporogenes	3	25-60
C. sporogenes	3	>200
C. tertium	1	100
C. welchii A (2), F (1)	3	1.5-6
C. welchii A	1	100

Table 2b. Antimicrobial Spectrum of Nisin Against Bacillus

Species	Number of strains tested	Sensitivity, units/ml
Bacillus carotarum	4	50-200
B. carotarum	3	>200
B. circulans	3	>200
B. coagulans	7	50-200
B. coagulans	4	>200
B. coagulans	3	3.2-12.5
B. licheniformis	3	>200
B. megaterium	2	0.7
B. polymyxa	3	25-200
B. polymyxa	1	>200
B. pumilus	8	25-200
B. pumilus	2	>200
B. subtilis	2	6
B. subtilis	8	25-100
B. subtilis	4	100-200
B. subtilis	2	>200

Table 2c. Antimicrobial Spectrum of Nisin Against Nonsporeformers

Species	Number of strains tested	Sensitivity, units/ml
Lactobacillus bulgaricus	1	0.35
L. casei	1	12
L. fermenti	6	15
L. helveticus	1	0.5
L. lactis	1	50
L. plantarum	1	30
Micrococcus candidus	1	>200
M. varians	1	>200
Faecal streptococci	23	>200
Staphylococcus pyogenes var. *aureus*	1	6
S. aureus	1	12
S. aureus	1	50
S. aureus	1	100
S. aureus	6	200

Except for *Haemophilus* (*Bordetella*) *pertussis* no other Gram-negative organisms tested have been found to be sensitive to nisin. These include: Pseudomonas, Salmonella, Shigella, Coliforms, Proteus, Pasteurella, Alcaligenes, Brucella, and Neisseria.

in the use of nisin in cheeses and concluded that it would be impossible to set a statutory limit to the amount which could be present in view of the variable amount of nisin which may be present naturally.

"Crystalline nisin has now been produced and a preparation standardized at an activity of 1,000,000 units/gm is being manufactured for use in food processing [26,28]. It is said to be prepared by growing the organism in sterilized separated milk, and that no other chemical than sodium hydroxide and sodium chloride are used in its manufacture or extraction." Nisin cannot be used indiscriminately in any type of food because of its narrow spectrum of antibacterial activity (Table 2). It has no activity against the common spoilage organisms of fresh food, e.g. Pseudomonas, and may even be destroyed by some groups of bacteria. Its most suitable application is to inhibit spores in processed foods where the vegetative forms of bacteria have been destroyed [29]. For reasons which are not fully understood nisin has been shown to lower the heat resistance of spores of even the more resistant strains of bacteria. Although many strains of *Clostridium botulinum* are sensitive to nisin, a few are resistant so it would be undesirable to use nisin to lower the heat treatment of foods below that required to destroy *C. botulinum* spores in foods where a *botulinum* hazard exists. Most canned products are subject to spoilage by thermophilic bacteria and it is for the control of these organisms that nisin is particularly effective. The concentration added before processing is generally of the order of 100 units/g, i.e., 1 g of 1,000,000 unit powder/10 kg. A comprehensive list of proposed uses for nisin is given in the Report of the Antibiotics Panel [4].

The panel concluded that no hazard existed in the use of nisin in cheeses or in products such as canned fruit providing the pH of the product was below 4.5, or where the product needs in any case to be processed under conditions which ensure the destruction of all mesophiles.

PLANT DISEASE CONTROL

The only antibiotic used commercially in Great Britain for plant disease control at the present time is griseofulvin. It is used in the control of Botrytis (grey mold) of lettuce. Only a small amount is sold each year for this purpose and the Ministry of Agriculture, Fisheries, and Food issued its approval in September 1957.

A recent increase in bacterial plant disease in Great Britain, however, gives indication of further developments in the future.

The presence of fire-blight, an extremely destructive bacterial disease of pears and apples, was confirmed in Great Britain in 1958 [30]. This was the first record of the presence of this disease in Europe.

Fire-blight is endemic in North Amerca, where it has been known for more than 150 years. Elsewhere it is found only in New Zealand. Within the past two years the disease has spread throughout southeastern England and has become a threat to the commercial production of pears.

One of the major problems in the control of this disease is the fact that it infects a large number of ornamental trees and shrubs in addition to the commercial pear and apple. Thus, any organized plan of eradication must include the susceptible trees and shrubs of cities, private residences, and farms.

In Great Britain the measures taken up to now have been directed toward total eradication. This means finding and destroying all infected trees and shrubs wherever they may be. The immensity of the problem is ilustrated by the fact that in the town of Southend-on-Sea, some 1745 noncommercial trees and shrubs have been removed and destroyed with approximately 9000 more logged for destruction [31]. This total was just in the current year in one city.

It would appear to some observers that such a program will ultimately become too costly to be feasible and a method of control, living with the disease, will have to be substituted.

In the United States antibiotic spray treatments are used prophylactically and therapeutically in the orchard as part of a routine disease control program along with insecticides and fungicides. The antibiotic used currently for fire-blight is streptomycin, occasionally in combination with oxytetracycline.

Such a procedure seems imminent for the pear grower of Great Britain. Since this constitutes a nonmedical use of antibiotics, another modification of the therapeutic substances act was required. Such action has already been taken and in Great Britain, in 1958, the Therapeutic Substances Statutory Regulation No. 614 permitted the use of streptomycin and oxytetracycline dust and sprays in plant disease control. Although this statutory regulation was promulgated two years ago there has been no use of antibiotics in this manner because of the program of eradication to which the government is committed. However, the spread of fire-blight seems to forecast the use of antibiotics in plant disease control in Great Britain in the near future.

SUMMARY

Table 3 shows the estimated total annual use of antibiotics for all purposes in Great Britain. This represents approximately 10% of the total used in the United States.

In summing up our subject we find several observations that characterize the British scene. Nonmedical use of antibiotics has been under a go-slow attitude since it inception. There appear to be several conditions which account for this.

A cautious attitude is prevalent among government officials which is based primarily upon medical experience with the untoward effects of antibiotics.

There is a lack of need for routine therapeutic levels in livestock feeding. Whereas in the United States much therapeutic feeding occurs at levels of 200 ppm and higher, this is not necessary in Great Britain. The pattern of poultry disease in Great Britain is less intense and is primarily enteric; in the United States it is primarily respiratory. Also, the general intensity of poultry production is lower and brings about over-all reduction in the disease level compared with conditions in the United States [32].

Table 3. Estimated Use of Antibiotics in Great Britain

Use	Amount, kg
Growth stimulation (feeds)	
Pigs	19,000-20,000
Poultry	19,000-23,000
Miscellaneous	1000
Veterinary therapy	1500
Human therapy	75,000-100,000
Plant disease control	Experimental only
Food preservation	Experimental only
Total	111,000-145,000

There is a suspicion in Great Britain that benefits of antibiotic feeding are only temporary.

Finally, and most important, is the recent increasing list of authoritative investigations indicating the development of antibiotic-resistant bacteria in the environment where antibiotics are used as feed supplements.

There is, however, an indication that food preservation with antibiotics may develop to a great degree. The clear-cut data that nisin, an antibiotic discovered and developed in Great Britain, occurs naturally in foods will surely stimulate its acceptance and perhaps show the way for others to follow.

ADDENDUM

In a joint memorandum, announced after preparation of this manuscript, the Ministry of Health and the Ministry of Agriculture, Fisheries, and Food recommended approval of nisin in food preservation essentially as promulgated in reference 4. At the same time this group also approved chlortetracycline as a fish preservative at a concentration not to exceed 5.0 ppm.

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THE FATE OF SOME ANTIBIOTICS APPLIED TO PLANTS

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INTRODUCTION

For an antibiotic to be an effective plant therapeutant, it should be absorbed by roots, stems, leaves, flowers, and fruits following application of relatively low concentrations of 100 ppm or so. To be most effective the compound should be translocated readily both upward and downward in disease-controlling amounts and after translocation should retain its antimicrobial activity for several weeks. Needless to say, we have not found an antibiotic that meets all these requirements. However, therapeutically effective antibiotics that are readily absorbed are translocated upward in the plant and retain half of their activity (half-life) for 1 to 3 weeks have been reported. The desirable but not essential requisite for practical plant-disease control by therapy, downward translocatability following application of low concentrations to plants, is, for the most part, absent.

Although antibiotics generally are not translocated downward in plants, many other organic compounds are. Urea, glutamic acid, glutamine, alanine, asparagine, fructose, glucose, sucrose, 2,4-dichlorophenoxy acetic acid, and the many products of photosynthesis are but a few of such reported compounds [1-5]. Several plant-growth regulators are notable because of their unique characteristic, after their downward transport, of being exuded from roots into soil without chemical change [6,7].

The pattern of distribution of many antibiotics in plants is so regulated by xylem and phloem transport that accumulation of applied therapeutics begins in leaves soon after their application to roots or stems [1,8,9]. Further, antibiotics absorbed by roots and translocated to leaves or those absorbed directly by the leaves following foliar application are not readily removed from these leaves [1,8].

The rate of inactivation following foliar application is an important factor in the use of antibiotic compounds for plant therapy. Inactivation may be almost complete before the compound is absorbed by the leaf or may not occur until after absorption. Inactivation associated with the leaf may be regulated by the following factors: (a) stability of the compound on the leaf surface, (b) concentration of compound applied, (c) amount that is subsequently absorbed, and (d) plant species involved.

Some antibiotics, notably those of the polyene group, which are inactivated while still on the surface of leaves, hold little promise in plant therapy. For example, pimaricin was almost completely inactivated within 3 hr when sprayed on bean leaves [10]. The surface inactivation of pimaricin was thought to be the result of oxidation or ultraviolet exposure.

Antibiotics absorbed by the leaf may also be inactivated rapidly. The antibiotic F-17, was altered to such an extent in bean leaves after 5 days that all its therapeutic activity was lost [1]. Similarly, absorbed griseofulvin in broad bean lost half its activity after only 4 days and filipin lost almost all its activity in apple leaves in 3 days [12].

Metabolic inactivation of some absorbed compounds, however, may proceed at a much slower rate. For instance, disease-controlling amounts of cycloheximide applied to cherry leaves were still present within the leaves after 3 weeks [13]. Other research resulted in the recovery and identification of this compound in wheat seedlings 5 weeks after application [14]. Some streptomycin was found unchanged in tobacco leaves 5 weeks after spray application [15].

This rate of metabolic inactivation depends to a certain extent on the plant species involved. For example, while vancomycin was completely inactivated in tomato and tobacco plants within 2 days, in cucumber some of the antibiotic was unaltered for at least 10 days [16].

The amount of antibiotic applied to the plant is also important and influences the effective period of the compound. When applied in amounts that greatly exceed those required for therapy, cycloheximide, streptomycin, and vancomycin were detected in plants for extended periods, the maximum reported being over a year [13, 16-18].

Although the number of effective antibiotics presently available for plant-disease control is not high, several are commercially used in crop production. Notable among these are streptomycin, used for fire blight control, and cycloheximide, used therapeutically for control of white pine blister rust.

What factors are involved in the apparent variation in rates of inactivation of absorbed therapeutics and in their limited translocation Obviously, antibiotics such as cycloheximide, helixin, antimycin, and others that are phytotoxic interfere with normal metabolism of the plant and as a result may be inactivated. However, vancomycin is reported to be nonphytotoxic even at the very high spray concentrations of 1000 and 10,000 ppm. Certainly in the case of vancomycin, and for all antibiotics for that matter, reasons for varying rates of decay within plants must be elucidated more specifically.

One or more of the following modes of inactivation within plants have been proposed for the various antimicrobial agents [9, 19]: (a) chemical combination of a metabolite with the antimicrobial agent resulting in a new compound that possesses little or no activity. This effect is frequently observed with the amino acid, cysteine, highly reactive because of the SH group which readily reacts with many antibiotics. (b) Loss of antimicrobial activity as a result of competitive inhibition of the antibiotic by an enzyme. (c) Inactivation through degradation as a result of hydrogen-ion concentration within the plant to which the antimicrobial agent is unstable. (d) Enzymatic degradation of the antibiotic. These four metabolic modes of inactivation are, of course, just a few of the possible mechanisms involved in determining the fate of therapeutants within plants.

Loss of antibacterial or antifungal activity of a number of antibiotics within plants seems to be explained, at least tentatively, on the basis of metabolic inactivation. Certainly metabolism must play an important role in the loss of activity of compounds such as penicillin, penicillic acid, and griseofulvin in plants. Metabolic inactivation in plants is perhaps best illustrated by the widely different half-lives of cycloheximide applied to the foliage or fruit of cherry. Rapid inactivation of this antibiotic occurred in cherry fruit, its half-life being only about 24 hr [20]. In the cherry leaves, however, the cycloheximide half-life is in excess of 3 weeks [7]. It has been suggested than an enzyme system in mature fruits causes the rapid loss of antifungal activity.

The inability of plants to translocate antibiotics downward, even those possessing long half-lives, may be related to varying rates of inactivation within the different tissues. The antibiotic, although antimicrobial in nonvascular tissue, may be inactivated rapidly as it moves deeper into the plant toward the phloem or only when within the phloem, so that the concentration of unaltered antibiotic within the phloem tissue never reaches a threshold level which permits its downward transport in effective amounts. However, metabolic processes are not the only ones that may limit the downward transport of antibiotics. For example, since the cellulose of plants is assumed to be negatively charged, the positively charged antibiotics such as streptomycin, the polypeptide antibiotics, and tetracyclines may be adsorbed to cell walls and thus prevented from moving. These are two of several possible reasons that may explain the absence of downward transport.

Economical plant therapy may become a reality in the near future. This use of antibiotics for disease control will result from learning to control the fate of these compounds and the discovery of new ones that are much more effective in control of diseases affecting the production of important crops. Perhaps one of the newer antibiotics reported in this conference, when evaluated as a plant therapeutic, might be 50 to 100 times as effective against plant diseases as those already known.

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EFFECTS OF THE TETRACYCLINE ANTIBIOTICS, THEIR DEGRADATION PRODUCTS, DERIVATIVES, AND SOME SYNTHETIC ANALOGUES ON THE GROWTH OF CERTAIN PLANT SYSTEMS

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INTRODUCTION

Since the beginning of the "antibiotic era," these compounds have been tested to determine their effects on the growth and metabolism of green plants. Originally such tests were run to determine the phytotoxicity of the antimicrobial agents in order to assess their possible use for the control of plant diseases. When references started to appear concerning their stimulatory effects on plants, the suggestion was made that definite data on the mode of growth action might best be obtained by using small aquatic plants capable of being rendered axenic, such as members of the Lemnaceae [1]. The value of these tiny flowering plants as test objects has been stressed many times in the plant physiological literature [2-4].

Following this suggestion, our original work with duckweed compared the activities of several antimicrobial compounds [5]. We found that the three related antibiotics, oxytetracycline, tetracycline, and chlortetracycline, could be distinguished by their effects on the growth of a duckweed (Lemna minor) under certain conditions. Time studies showed that solutions of the antibiotics which had aged for several weeks caused more stimulation than fresh solutions, or, caused stimulation when the fresh solutions were toxic [5]. This led us to suspect that the antibiotics were degrading, under our test conditions, to other compounds, one or more of which was the cause of the stimulation. At this time intensive study was in progress in determining the structures of the tetracyclines, making degradation products, derivatives, and synthetic analogues available. Tests evaluating many such compounds confirmed the fact that several of the degradation products were extremely stimulatory.

It should be pointed out before going further that the test conditions set up for this purpose, detecting subtle differences in organic molecules, are quite different from those used in many physiological experiments. This is particularly true of the basal medium. The one we use is not optimal because we are interested in intermediate as well as extreme effects, both inhibitory and stimulatory. However, mode of action studies should be and are carried out under a variety of experimental conditions.

Our primary test system is Lemna minor, grown axenically and under the conditions described below. Other test systems used for confirmatory and comparative purposes include the duckweeds Lemna gibba, Spirodela polyrrhiza, Spirodela oligorrhiza, Wolffia columbiana, and the ferns Azolla caroliniana and Salvinia rotundifolia (all but the last in axenic culture). The conditions for the growth of these other plants were the same as those for Lemna minor, unless so indicated.

MATERIALS AND METHODS

The basal medium and the general techniques used in previous studies were employed throughout the investigations to be reported here [5, 6]. All compounds evaluated were made up at ten times the highest level to be tested, sterilized by sintered-glass filtration, and added aseptically to the sterilized basal medium in amounts to give the desired concentrations. A few test compounds, known to be stable, were added to the medium prior to sterilizing. All experiments were run in replicates of five and repeated, except in a rare case where an insufficient supply of compound was available. Experimental transfers for each test were made by placing an axenic rosette of four fronds in each test flask. All transfers for a given experiment came from one stock flask. Growth during the test period was in an air-conditioned culture room in continuous diffuse light, with temperature controlled at 22.5 C. Results to be given here are expressed on the basis of wet weights. The value of expressing growth in terms of frond multiplication for certain physiological purposes is recognized, as are other methods of expressing growth. We have all these types of data recorded and available for discussion purposes, but feel that for the objectives of this particular type of presentation wet weight is the most practical way of expressing our results. No compounds were included which were not water soluble because of the unusual results obtained with trace amounts of several organic solvents.

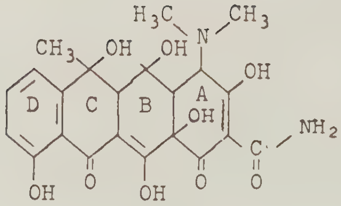
EXPERIMENTAL

The effects of the three tetracyclines (Fig. 1) on the growth of Lemna minor are shown in Table 1. At 3 weeks there is considerable toxicity at 10 and 20 ppm with all tetracyclines. At the lower levels of 1 and 5 ppm, differences can be seen, showing a graded response sufficient to distinguish the compounds. When the experiment runs for 8 weeks, the results are strikingly different from those found at 3 weeks. There is significant increase in growth in the presence of oxytetracycline at 1-10 ppm and in chlortetracycline at 1 ppm, suggesting that breakdown products are involved. The explanation for the differences between the two antibiotics is probably twofold as will be discussed in detail in later sections. First, the plants were dead in the flasks containing 5 ppm chloretetracycline and above, and were probably killed during the first three weeks. As a result, the plants could not respond to any chemical changes which occurred subsequently. The plants were not dead at 10 and 20 ppm of oxytetracycline although strongly inhibited. Therefore, they were still capable of responding to a change in the chemical environment.

Second, under the pH conditions of this type of experiment, with the original pH around 5.0, oxytetracycline breaks down in a different manner than chlortetracycline, giving those products which have a favorable influence on growth. Even

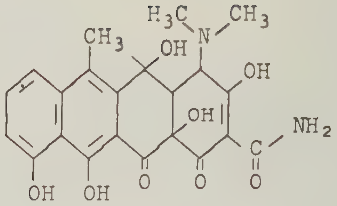
Table 1. Effects of the Tetracyclines on the Growth of Lemna minor

Antibiotic	Number of weeks	Change from control, %				Reference No.
		1 ppm	5 ppm	10 ppm	20 ppm	
Oxytetracycline	3	0	+10	−40	−75	19, 22
Tetracycline	3	−8	−60	−75	−80	18
Chlortetracycline	3	−10	−80	−85	−90	17, 29
Oxytetracycline	8	+40	+350	+80	−60	19, 22
Chlortetracycline	8	+190	−50	−94	−96	17, 29



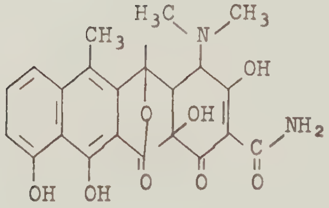
H3C
CH3
CH3
OH
OH
N
OH
D
C
B
A
OH
NH2
C
OH
O
OH
O
O

I
Oxytetracycline



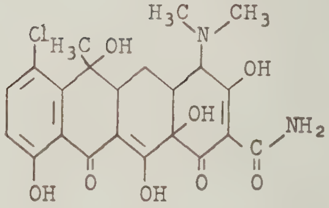
H3C
CH3
CH3
OH
N
OH
OH
NH2
C
OH
OH
O
O
O

II
Anhydrooxytetracycline



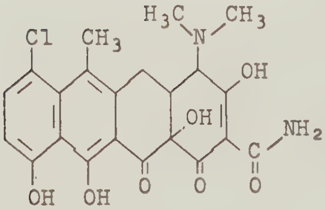
H3C
CH3
CH3
N
OH
O
OH
NH2
C
OH
OH
O
O
O

III
Apooxytetracycline



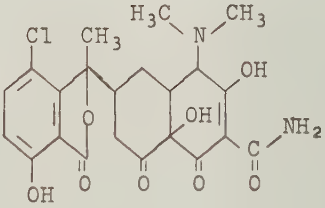
H3C
CH3
Cl
H3C
OH
N
OH
OH
NH2
C
OH
O
OH
O
O

IV
Chlortetracycline



H3C
CH3
Cl
CH3
N
OH
OH
NH2
C
OH
OH
O
O
O

V
Anhydrochlortetracycline



H3C
CH3
Cl
CH3
N
OH
O
OH
NH2
C
OH
O
O
O
O

VI
Isochlortetracycline

VII = IV, Cl = H, Tetracycline
VIII = V, Cl = H, Anhydrotetracycline
IX = VI, Cl = H, Isotetracycline

Fig. 1.

though certain products of chlortetracycline are more active in stimulating growth, they are not formed as rapidly under these test conditions.

Following the suggestion that the breakdown products are responsible for the growth effects, we tested the available isolated degradation products, derivatives and certain synthetic analogues to determine their effects on growth under our test conditions. The growth effects of some of the compounds which have particular bearing on the present discussion are shown in Table 2.

Certain facts emerge from these results which should be pointed out because they form the basis for the discussion which follows: The formation of anhydro-compounds causes a reduction in toxity; both apoisomers of oxytetracycline cause favorable growth effects, the beta form being significantly better; iso-compounds are the most striking in their effects on growth, that of chlortetracycline being markedly better than that of tetracycline.

Table 2. Effects of Certain Tetracycline Degradation Products, Derivatives, and Synthetic Analogues on the Growth of *Lemna minor* After Three Weeks

Compound	Change from control, %				Reference No.
	1 ppm	5 ppm	10 ppm	20 ppm	
Anhydrooxytetracycline	+15	+110	+175	+190	22
Anhydrotetracycline	+20	+80	−60	−90	18
Anhydrochlortetracycline	0	0	+15	+40	31,29
α-Apooxytetracycline	0	+10	+15	+70	21,22
β-Apooxytetracycline	+45	+125	+240	+200	21,22
Isotetracycline	+50	+145	+215	+310	16
Isochlortetracycline	+40	+220	+450	+700	30
5,12a-Diacetyloxytetracycline	+25	+25	+65	+65	22
3-Methyl-7-hydroxyphthalide	+10	+50	+80	+225	20,26
2-Acetyltetralone-1	+10	+215	+175	+100	23
Desdimethylaminoanhydro-oxytetracycline	+30	+75	+25	−40	22
Desdimethylaminoanhydro-tetracycline	0	−25	−45	−60	24
Desdimethylaminoanhydro-chlortetracycline	−20	−70	−75	−90	29

DISCUSSION

We have shown that when the tetracycline antibiotics are incorporated into a basal medium supporting suboptimal growth of duckweed at levels from 1 to 20 ppm, after three weeks there is generally an inhibition of growth, except at the lowest level. Also there are quantitative differences among the effects of the three antibiotics. At the lower levels we have shown that, over a growth period of 8 weeks, the end result is a significant increase of growth over control plants. It is postulated that this is due to the breakdown of the antibiotic to compounds more favorable to growth.

Evaluation of degradation products, derivatives, and synthetic analogues of the starting test compounds shows several of these to have striking growth enhancement. This is particularly true of β-apooxytetracycline and of isochlortetracycline.

We believe that the following is the explanation of the results obtained under our test conditions, considering the pH, stability of the antibiotics, and what is known about their chemical degradation. Under acid conditions oxytetracycline breaks down to the anhydro form, which as has been shown, is less growth inhibitory than the parent molecule. The anhydro form immediately breaks down in turn to a mixture of α- and β-apooxytetracyclines. The β isomer is more stimulatory and the observed effect is probably due to this compound.

Under these same conditions chlortetracycline breaks down primarily to anhydrochlortetracycline which is relatively stable. Another of its breakdown products, isochlortetracycline, is extremely stimulatory to the growth of duckweed under our test conditions. Alkaline conditions favor the formation for the iso-compound; since the pH of the basal medium is on the acid side, the formation of the iso-compound is slow, resulting in a final growth effect which is not nearly as striking as that obtained by using isochlortetracycline as the starting material.

This is probably why, under these conditions, oxytetracycline causes a greater increase in growth and why this increase occurs sooner than with chlortetracycline.

POSSIBLE MODES OF ACTION

We have previously suggested that chelation is an explanation for the mode of stimulatory action of antibiotics in the growth of plants [5]. More general statements have been made in the same vein by others [7, 8]. The tetracyclines have strong chelating properties [9] and, in fact, these properties are utilized in the methods of isolation of these compounds. The unusual chelating abilities of the parent molecules probably explains the toxicity as the binding of all the heavy metals in solution. The formation of the anhydro, apo and iso compounds results in substances having different powers of metal chelation more suitable to the optimal growth of the test material in this case.

The most powerful chelating portion of the tetracycline molecule resides in the β-diketone system of the B and C rings [10]. When this is significantly changed, as does occur in the formation of the anhydro, apo and iso compounds, the new molecular structures have a more favorable effect on growth.

The fact that the growth effects could not possibly be ascribed to their use as nutrients is best shown by the amount of nitrogen, for example, which is several orders of magnitude less than optimal for growth. We do not plan to discuss whether the action could be described in terms of auxin physiology in this case, as the whole question of modes of action of plant auxin and implication of chelation in this is currently being discussed elsewhere [11-15]. For this reason and because of lack of time allotted for the present paper, we shall not attempt a critical discussion of our experimental data; however, such a discussion will be presented in a subsequent publication.

In brief, we believe that the chelating properties of the antibiotics and their products are the most important ones in explaining antibiotic growth-effects in plants. However, we also believe that it would be asking too much of a molecule to expect, in a complex biological system, that its ability to chelate with a metal (or metals) could account for all of its biological properties. A good example of why we feel this way is a comparison of the effects of isotetracycline and isochlortetracycline. Both cause a dramatic increase in growth, yet growth caused by isochlortetracycline is significantly greater than that caused by isotetracycline. However, to the best of our knowledge, the difference in the molecules has no connection with their chelating properties.

These data, in conjunction with well documented symbiotic interrelationships between microorganisms and higher plants, have suggested to us a possible answer to the enigma of the formation of antibiotics by microorganisms. When we asked ourselves previously, "What is the function of an antibiotic in nature?", we usually thought of the all-important therapeutic properties of these molecules. Might this be only a transitory intermediate stage? Is the purpose to produce a molecule which will then be available in the soil in a different final form, affecting the growth of plants?

It would then follow that antibiotics are not isolated as such from the soil since they are not the end products present in the soil. Our present test systems, both practical and theoretical, are geared to pick up that biological property expressed by the inhibition of microorganisms. Perhaps a different picture might emerge if the test systems were designed to evaluate biological responses other than antibiosis.

SUMMARY

1. A biological system based on the growth of small aquatic plants in a liquid synthetic medium is utilized for distinguishing small chemical differences in organic molecules. In the present discussion it is applied particularly to the tetracycline antibiotics and related compounds.

2. Differences in growth effects for a single antibiotic, with time, have been shown to be due to degradation of the parent molecule.

3. The observed phenomena can be correlated with the changes in chelating properties of the compounds concerned.

4. Although a dominant role is postulated for chelation in the observed growth effects, it is not felt that chelation is sufficient to account for all of the biological effects demonstrated.

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DISCUSSION

Q: In connection with the chelation, have you any knowledge of the effectiveness of tetracycline?

DR. NICKELL: I would have to look it up in our data book. It is not one that we included in the results to date.

DR. PETERSON (Texas): Do you know if any breakdown occurs in the products?

DR. NICKELL: I would doubt it, because this is all in solution. Remember, we are dealing in higher plants and we are dealing in terms of weeks for an experiment, not days, so when we say something breaks down, we are putting it under conditions where we want it to break down, because we think that this is what happens. In three weeks we get very little effect. In eight weeks we get the effect, so by this roundabout reasoning, I would suggest that there probably is not much if any, of these things present intially.

Q: The point I was trying to make is that the breakdown in association with the higher plant is important. Is it possible that any of the breakdown products are biologically active with respect to microbes?

DR. NICKELL: The iso form, isochlortetracycline, which is the most powerful in its growth effect, has no antimicrobial activity and the apo forms are practically inactive too.

Q: I wondered about the technique you used to detect the derivatives.

DR. NICKELL: Most of the ones that we have discussed here are very readily determined both by their change in fluorescence and paper chromatography, and, of course, with the residual parent molecule, the antimicrobial activity. The ones which are most active for our purposes have essentially no antimicrobial activity.

DR. GOODMAN (University of Missouri): Have you ever tried to get a simulated effect on microorganisms?

DR. NICKELL: Yes, and some fungi, when grown on a synthetic medium, not in a complicated medium with lots of unknown material, definitely respond rather markedly to some of these materials. It is strictly an increase in growth based on wet and dry weight.

THE PHARMACOLOGY OF TYLOSIN, A NEW ANTIBIOTIC, IN THE CHICKEN

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DESCRIPTION

Tylosin is an antibiotic fermentation product of a newly described isolate of Streptomyces fradiae, an actinomycete. The antimicrobial spectrum has been described [1] as essentially Gram-positive. In vitro it is very active against avian pleuropneumonia-like organisms (PPLO), swine PPLO, and spirochetes. In vivo tests have shown activity against Leptospira sp. in laboratory animals and swine, erysipelas of turkeys and swine, avian PPLO, and Hemophilus pertussis. Activity against avian coccidia and several intestinal parasites has been reported [2]. Activity against large viruses such as those producing infectious synovitis of chickens, meningopneumonitis of mice, ornithosis, and trachoma has been demonstrated in animal or tissue-culture tests.

The chemistry of tylosin will be described in a future publication [3].

AGRICULTURAL USES

Tylosin is of particular interest in veterinary medicine because of its unusual activity against avian PPLO producing chronic respiratory disease of chickens and turkeys [4, 5, 6]. Tylosin also has growth-promoting properties in both chickens and swine.

Figure 1 summarizes the results of several experiments demonstrating growth-promoting properties of tylosin in chickens [7, 8]. In these experiments tylosin was compared to control and procaine-penicillin treatments. In experiments 1, 3, and 4, tylosin produced an increase in eight-week weights and improved feed efficiency. In experiments 5, 6, 7, and 8, growth-promoting properties were not as evident; there was a slight improvement in feed efficiency over the penicillin-treated birds.

The effects of tylosin on growth rate and feed efficiency of swine have been reported [9]. Improvements of 8.3% in average daily gains and 1% in feed efficiency over control groups were obtained by feeding tylosin at a level of 40 g/ton of complete feed from weaning to 40 lb, 20 g from 40 to 100 lb, and 10 g from 100 lb to market weight (Fig. 2).

PHARMACOLOGY

Toxicity. Because of the unusual activity of tylosin against the PPLO producing chronic respiratory disease of chickens and infectious sinusitis of turkeys, pharmacological studies were conducted with particular reference to toxicity, absorption, and excretion of tylosin in the fowl.

Acute toxicity studies of the tartrate salt of tylosin established the following LD_{50}'s in chickens: oral, 5400 ± 175.8 mg/kg; i.m., 432 ± 7.4 mg/kg; and i.v.,

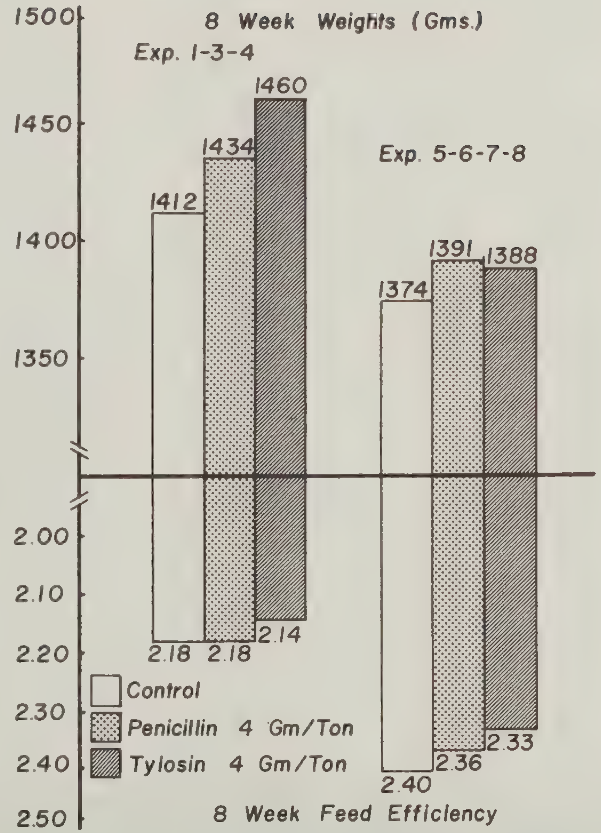

Fig. 1. Effect of tylosin and procaine penicillin on average daily gain and feed efficiency of chickens for an eight-week period.

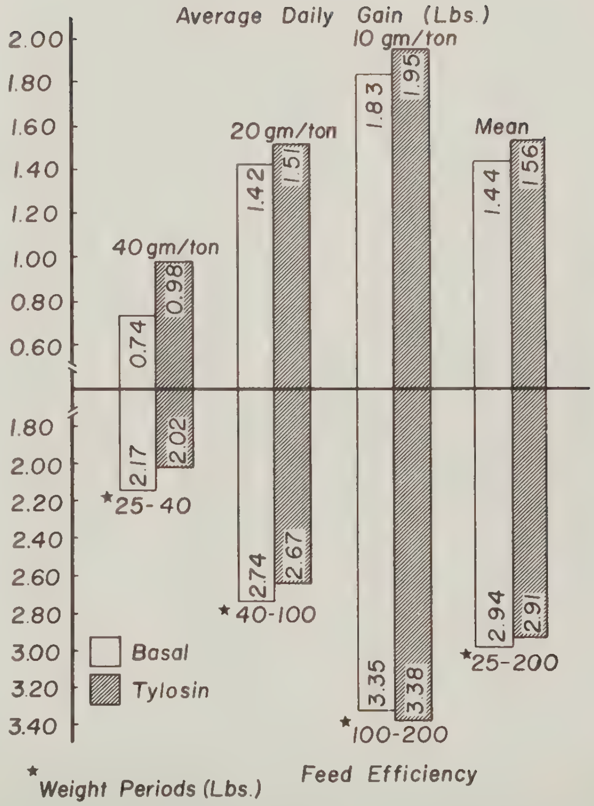

Fig. 2. The effects of tylosin on growth rate and feed efficiency of growing-finishing swine, by weight periods.

58.6 ± 4.14 mg/kg. For these experiments 162 five-week-old Smitherman-cross broiler-type cockerels were used.

Table 1 compares the acute oral toxicity of tylosin to several other antibiotics in various species. Note that tylosin has a wide margin of safety in all species studied and compares very favorably with other antibiotics. Penicillin is exceptional in that it is essentially nontoxic, except in the guinea pig.

Table 1. Acute Oral Toxicity of Several Antibiotics, mg/kg

Antibiotic	Animal				
	Mouse	Rat	Dog	Chicken	Guinea pig
Tylosin base	>5000	>5000	>800	2122	
Tylosin tartrate	>6200	>6200		5400	>1000
Chlortetracycline	1500	>3000	750		
Oxytetracycline	6696				
	7200				
Streptomycin	9000				
Penicillin	Essentially nontoxic except in guinea pigs				
Bacitracin	>3750				

Subacute studies were conducted in rats and dogs [10]. Rats receiving subacute dosages of tylosin as high as 100 mg/kg daily for one month showed no ill effects other than occasional diarrhea. In another study, dosages of tylosin up to 1000 mg/kg administered subcutaneously for 28 days did not affect weight gains or blood values in rats. Dogs given up to 100 mg/kg orally for 30 days showed no ill effects.

Subacute toxicity studies in chickens have been carried out to substantiate the data available from laboratory-animal studies. Criteria in these studies were weight gains and feed efficiency, clinical pathological studies, and gross and microscopic examinations. In these experiments, groups of 12 five- to seven-week-old Smitherman-cross cockerels were given 30 daily intramuscular injections of 5, 25, 100, or 200 mg/lb. Birds treated with 5 mg/lb showed an increased rate of gain as compared to control birds. At 25 mg/lb, gains were equal to the controls and at 100 and 200 mg/lb, weight gains were decreased. Death occurred at the 200 mg/lb level. The high levels produced mild liver and kidney degeneration. In another study tylosin was administered in the feed to one-day-old, unsexed White Rock chickens at levels of 200, 500, 1000, and 3000 g/ton for four and one half months. No abnormal values were found in hemoglobin, hematocrit, white blood cells, thrombocytes, sedimentation rates, coagulation time, or differential counts. All tissues were grossly and microscopically normal. Figure 3 compares the weight gains of control and treated birds. Each group consisted of two replicates of ten birds each. Note that the control group showed the lowest rate gain although the differences between all treatments could not be shown statistically. No depression of feed consumption or weight gain, even at 3000 g/ton, was found. Figure 4 compares the feed conversions of these birds over the four and one half month period. All groups performed in comparable fashion.

Blood levels. All microbiological values reported were obtained with a cup-plate assay using Sarcina lutea.

Average blood levels following administration of a 25-mg/kg subcutaneous dose to eight eight-week-old Smitherman-cross cockerels are shown in Fig. 5. Peak levels occurred at 15 min and declined to 0 by 24 hr. Figure 6 shows the blood levels produced following oral administration of single or repeated dosages

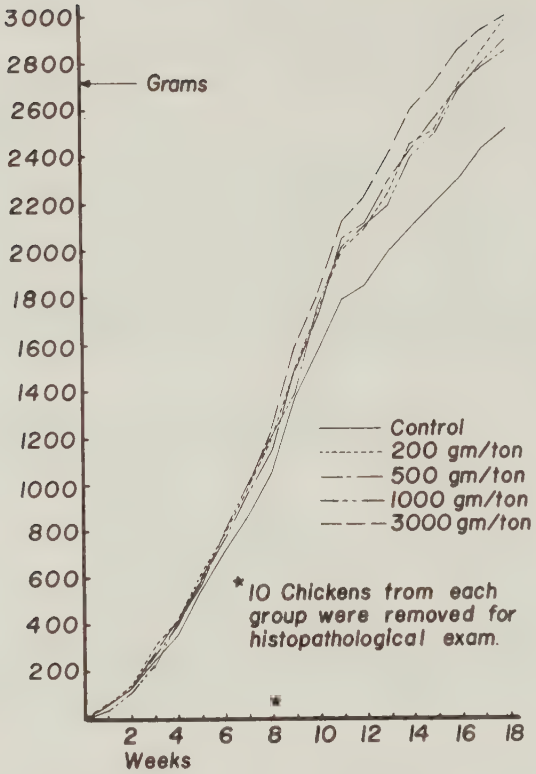

Fig. 3. Mean weight gains of chickens fed tylosin tartrate over an 18-week period.

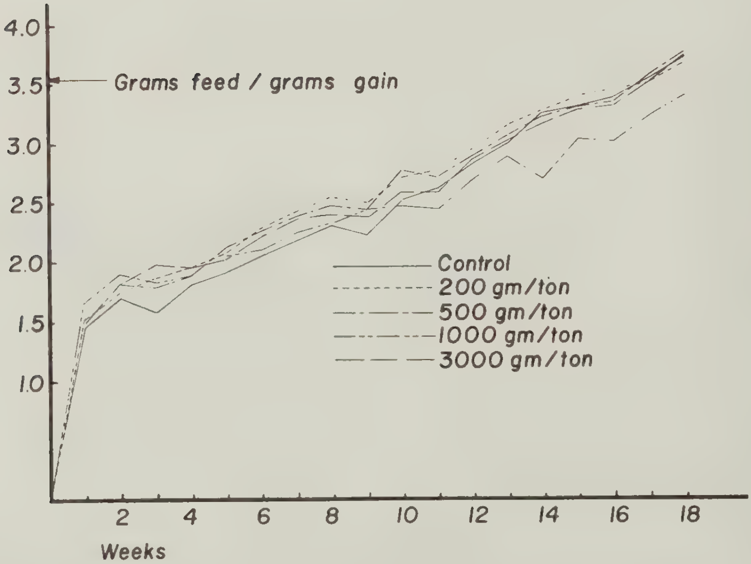

Fig. 4. Feed conversions of chickens fed tylosin tartrate over an 18-week period.

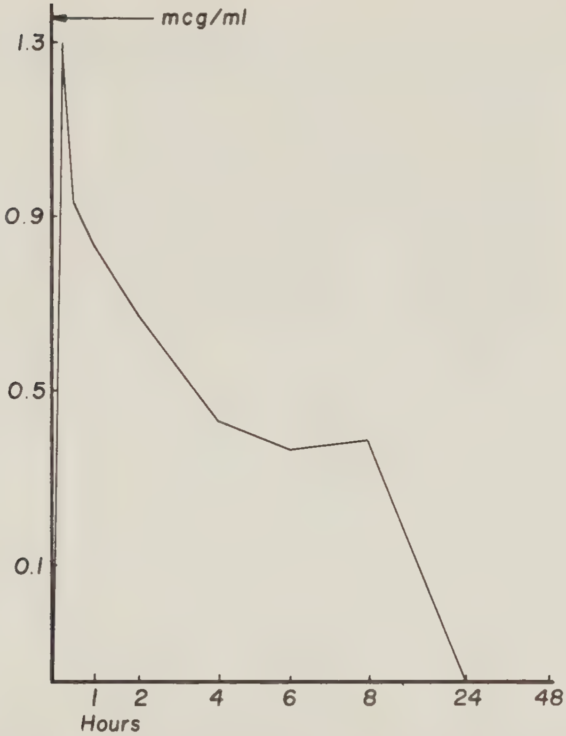

Fig. 5. Mean microbiological activity of chicken sera following administration of a 25-mg/kg subcutaneous dose of tylosin tartrate.

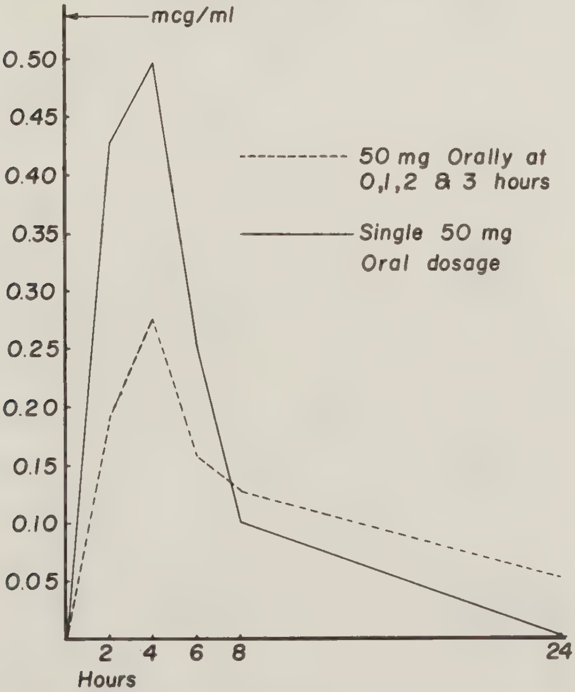

Fig. 6. Mean tylosin activity in chicken sera following single or multiple oral doses of tylosin tartrate.

Table 2. Results of Microbiological Assay of Urine and Feces After Treatment with Tylosin Tartrate

Dosage, mg/kg	Route	Total dosage, mg,	Concentration										
			Urine, μg/ml							Feces, μg/g			
			2 hr	4 hr	6 hr	8 hr	24 hr	48 hr	72 hr	8 hr	24 hr	48 hr	72 hr
25	IM	45.3	30.5	2.5	1.7	0.9	0.8	—	1.2	40.1	3.7	< 0.4	< 0.4
		57.3	60.0	30.7	13.4	2.5	0.9	—	—	75.6	3.4	1.2	—
		47.1	27.7	15.3	2.6	2.2	0.4	—	—	86.2	7.4	0.7	—
		49.6	340.0	333.0	225.0	152.0	1.3	1.1	0.3	NS	189.0	8.9	3.3
		50.4	380.0	220.0	140.0	37.5	33.3	33.3	0.5	200.3	136.1	2.9	9.0
		52.5	462.0	53.5	20.4	19.8	23.4	2.0	0.5	317.5	128.5	20.8	3.1
100	IM	150.0	240.0	55.0	30.7	31.50	10.0	2.1	1.1	619.0	245.7	160.1	81.3
		215.5	680.0	275.0	84.0	63.8	33.0	3.2	0.8	293.0	115.3	44.4	3.6
		170.5	740.0	310.0	64.0	88.0	84.5	5.8	1.2	1927.8	831.6	182.4	12.3
		169.5	1270.0	900.0	358.0	355.0	119.0	4.2	1.5	307.1	217.4	125.7	189.0
		243.0	273.0	79.0	25.6	30.8	43.0	6.2	1.1	434.7	274.1	138.9	17.0
		186.0	758.0	335.0	153.0	26.0	70.0	8.8	1.2	708.6	146.5	20.3	6.5
25	Oral	26.8	0.16	1.9	7.4	4.4	0.5	0.2	< 0.1	—	100.2	59.7	35.2
		26.9	85.0	45.0	17.5	9.2	0.5	< 0.1	—	326.0	14.1	1.1	< 0.4
		26.3	17.5	8.8	4.5	2.0	< 0.1	—	—	1096.2	29.5	6.1	< 0.4
		20.0	21.2	23.1	5.4	6.8	0.1	< 0.1	—	236.3	7.6	3.0	2.0
		21.1	10.0	4.2	1.9	0.8	0.1	< 0.1	< 0.1	157.8	4.9	9.0	1.9
		21.0	< 0.1	0.5	0.8	0.5	0.2	< 0.1	—	182.4	9.8	2.3	3.9

Table 2 cont.

Dosage, mg/kg	Route	Total dosage, mg,	Concentration										
			Urine, μg/ml							Feces, μg/g			
			2 hr	4 hr	6 hr	8 hr	24 hr	48 hr	72 hr	8 hr	24 hr	48 hr	72 hr
100	Oral	182.0	690.0	1560.0	117.0	22.8	173.0	0.7	—	2835.0	359.1	231.3	10.8
		104.5	690.0	1480.0	66.5	14.5	12.5	0.3	—	2192.4	255.2	26.7	3.2
		112.5	1660.0	1150.0	1050.0	1100.0	425.0	14.0	2.7	NS	737.1	35.7	14.7
		106.8	13.2	172.0	265.0	114.0	56.0	0.3	—	NS	1360.8	155.0	13.8
		105.0	218.0	295.0	330.0	35.5	7.4	0.4	—	1625.4	139.9	4.0	0.9
		101.5	3.5	7.9	1.1	0.4	12.7	0.1	<0.1	NS	491.4	43.1	12.9
250	Oral	592.5	3100.0	970.0	108.0	76.0	12.5	0.8	0.4	2324.7	103.9	172.9	10.3
		400.0	32.0	350.0	750.0	268.0	10.8	0.1	<0.1	1088.6	907.2	14.0	2.3
		352.5	1570.0	375.0	59.5	22.8	5.6	0.9	0.7	3194.0	308.1	52.7	45.4
		485.0	1350.0	1820.0	1810.0	1120.0	86.0	4.9	2.0	1766.6	1039.5	25.0	7.6
		425.0	2630.0	1750.0	700.0	218.0	37.5	2.9	0.4	2324.7	470.6	130.4	113.4
		407.5	71.0	192.0	96.0	108.0	42.0	2.2	0.2	1190.7	1190.7	111.5	4.7

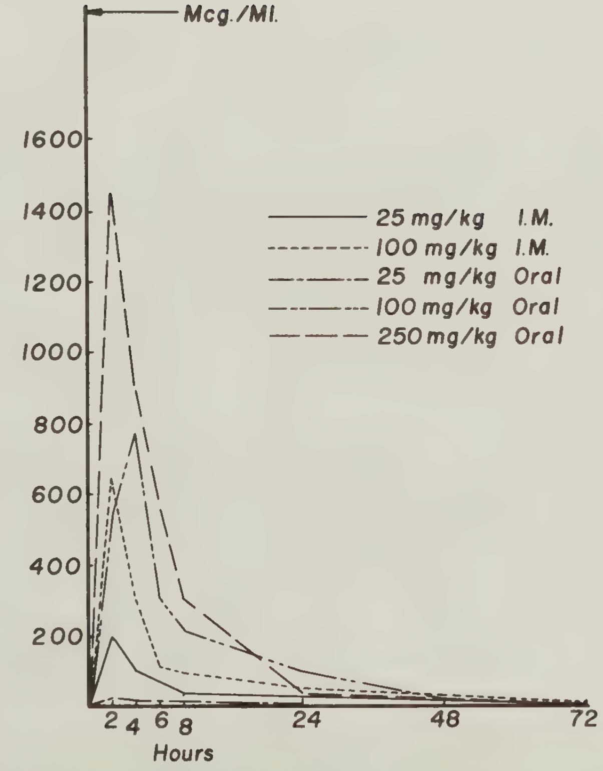

Fig. 7. Mean microbiological activity in chicken urine following oral or intramuscular administration of tylosin tartrate.

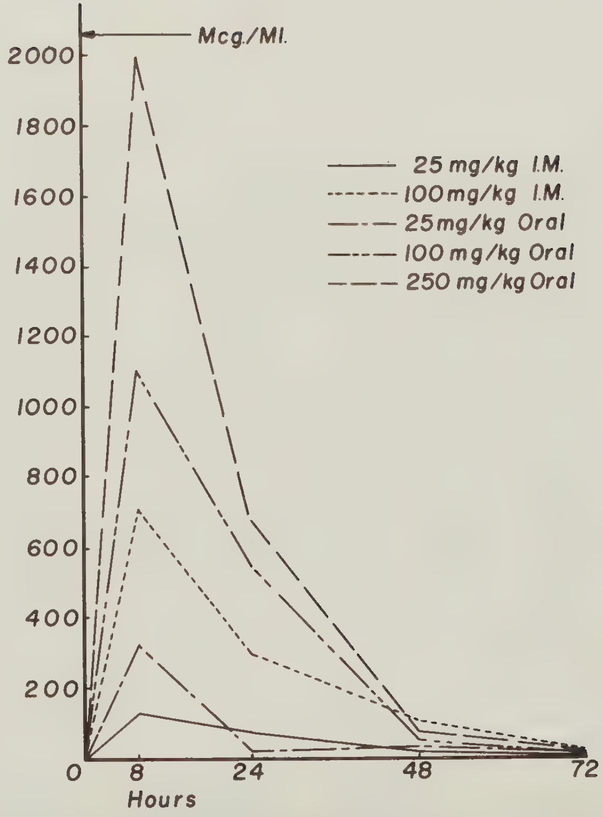

Fig. 8. Mean microbiological activity in chicken feces following oral or intramuscular administration of tylosin tartrate.

Table 3. Percent Recovery of Total Administered Dose of Tylosin Tartrate in Urine and Feces

Dosage, mg/ kg	Route	Total dosage, mg	Totals				
			Urine, µg	Feces, µg	Urine, %	Feces, %	Total %
25	Intra-muscular	45.2	1122.4	817.0	2.5	1.8	4.3
		57.2	426.1	1028.9	0.7	1.8	2.5
		47.1	108.2	664.3	0.2	1.4	1.6
		49.6	3451.0	5865.1	6.9	11.8	18.8
		50.4	5534.8	16996.4	11.0	33.7	44.7
		52.5	5206.6	17680.6	9.9	33.7	43.6
100	Intra-muscular	150.0	10469.2	11860.8	7.0	7.9	14.9
		215.5	7844.3	16724.9	3.6	7.8	11.4
		170.5	11019.1	41387.6	6.5	24.3	30.7
		169.5	10800.8	1412.3	6.4	0.8	7.2
		243.0	9220.3	38968.6	3.8	16.0	19.8
		186.0	17601.1	12245.1	9.5	6.6	16.0
25	Oral	26.7	116.4	1512.7	0.4	5.6	6.7
		26.9	1546.6	5380.5	5.7	20.0	25.8
		26.2	72.7	8662.5	0.3	30.0	30.3
		20.0	158.5	4170.7	0.8	20.8	21.6
		21.1	62.2	4930.0	0.3	23.3	23.6
		21.0	13.3	3893.5	0.1	18.5	18.6
100	Oral	182.0	24281.5	114127.6	13.3	62.7	76.0
		104.5	10302.7	51691.9	9.9	49.5	59.3
		112.5	29485.0	26502.0	26.2	23.6	49.8
		106.8	2018.1	5648.3	1.9	5.3	7.2
		105.0	4090.6	18926.1	3.9	18.0	21.9
		101.5	2006.4	9400.5	2.0	9.2	11.2
250	Oral	592.5	27491.5	101927.9	4.6	17.2	21.8
		400.0	5803.2	83313.2	1.4	20.8	22.3
		352.5	23461.8	31598.4	6.7	9.0	15.6
		485.0	44865.3	152613.3	9.2	31.5	40.7
		425.0	28340.8	144052.0	6.7	33.9	40.6
		407.5	6832.2	62873.2	1.7	15.4	17.11

of tylosin. A single dose of 50 mg of tylosin administered by stomach intubation to four 720-g chickens produced peak levels at about 1 hr followed by a decline to a negligible point at 24 hr. When repeated doses of 50 mg were administered orally at 0, 1, 2, and 3 hr to 15 2-kg birds, activity was prolonged to the 4-hr sample followed by a gradual decline to a negligible point at 24 hr. Some difficulty was encountered initially in obtaining reproducible blood levels following oral treatment in poultry possibly because of blockage of the absorptive sites or complexing of the antibiotic by the feed. In order to obtain consistent results, a system was evolved in which fasted birds were given dosages of antibiotic in a feed-slurry preparation in order to standardize the test and yet study a situation comparable to that in the field. This system has proven to be very satisfactory.

Excretion. The routes of excretion of tylosin were studied in surgically prepared birds in which urine and feces could be collected separately [11]. The anal ring was incised laterally and the cloaca sutured to the ventral surface of the tail, exposing the ureteral openings. A plastic disc was anchored over the ureteral openings for attachment of a rubber collection bag. A plastic collar was sutured over the posterior end of the bird for collection of the feces. Table 2 shows the activity in the urine at various time periods following treatment. Average concentrations ($\mu g/ml$) in the urine are shown in Fig. 7.

Dosages of 25 and 100 mg/kg intramuscularly and 25, 100, and 250 mg/kg orally were administered to groups of six birds. Assays were performed on urine samples collected at 0-2, 2-4, 4-6, 6-8, 8-24, 24-48, and 48-72 hr. Peak levels occurred in the 2- to 4-hr sample following both oral and intramuscular administration and declined rapidly thereafter.

Figure 8 depicts the average fecal levels ($\mu g/g$ wet feces) of these dosages of tylosin. Collection periods were 0-8, 8-24, 24-48, and 48-72 hours. The highest level occurred in the 8-hr sample in all cases. Activity was correlated with the route of administration inasmuch as more was recovered following oral treatment in every case.

Table 3 shows the recovery values for each of the six birds in each group. Total recoveries of antibiotic in the urine and feces following intramuscular treatment with 25 and 100 mg/kg ranged from 1.6 to 43.6%. Following oral treatment, total recoveries in both feces and urine ranged from 6 to 76%. Great variability in the amount of antibiotic recovered was apparent.

ACKNOWLEDGMENT

The authors wish to thank Mr. L. D. Baker, Mr. A. Branscum, Mr. G. G. Carter, and Mr. R. W. Rosebrock for their valuable technical assistance.

SUMMARY

The toxicity, absorption, and excretion of tylosin in the chicken has been studied. Acute, subacute, and chronic toxicity studies indicate that tylosin is a nontoxic antibiotic.

Blood levels, which were produced by subcutaneous administration of 25 mg/kg of tylosin, peak at 15 to 30 min and decline to zero by 24 hr. Orally, peak levels occur at about 2 hr and disappear by 24 hr.

Tylosin is eliminated through the urinary and biliary tract of the chicken. Attempted recovery of total dose was highly variable following both oral and parenteral treatment.

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TYLOSIN TREATMENT OF EXPERIMENTAL MYCOPLASMA GALLINARUM INFECTIONS OF CHICKENS AND TURKEYS

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INTRODUCTION

In the years 1942 through 1951 the ravages of chronic respiratory disease caused an estimated average annual mortality of more than 14 million chickens in the United States [1]. The losses undoubtedly have not diminished since that time. In addition, further losses occur from decreased weight gains, lowered feed utilization, extra time needed to bring broilers to market, increased rate of condemnation, and lower rate of egg production and hatchability.

Infectious sinusitis of turkeys with the often-present air-sac infection is also a very devastating disease. In a study conducted by the National Poultry Improvement Plan in 1953 sinusitis was cited as one of the four most important diseases of turkeys in 11 of the 19 leading turkey-raising states [2]. The mortality rate is usually highest when air-sac lesions are prominent but all infected birds suffer loss of appetite, reduced weight gains, and decreased feed utilization.

It is now generally recognized that pleuropneumonia-like organisms (PPLO) are the primary causative agent of infectious sinusitis and chronic respiratory disease [3]. Frequently bacteria such as Escherichia coli and Staphylococcus aureus invade the air sacs secondary to PPLO infection and cause more severe lesions. The viruses of Newcastle disease or infectious bronchitis are often found associated with PPLO in cases of CRD. However, the presence of the PPLO is necessary for either of these two conditions and the key to successful control of infectious sinusitis or chronic respiratory disease is control of the PPLO.

Properties of tylosin tartrate. Tylosin is a fermentation product of a newly described isolate of the actinomycete, Streptomyces fradiae [4]. The tartrate salt is readily prepared from tylosin base. Tylosin tartrate is stable in aqueous solution at room temperature in the pH range of 5.5 to 7.0 for at least one month. It is readily soluble in water in the order of 600 mg/ml and forms a clear colorless solution [5]. It is palatable to poultry and causes no noticeable distress when inhaled as a dust by chickens. Tylosin has a wide margin of safety in all species that have been examined [6].

The antimicrobial spectrum of tylosin is essentially Gram-positive and in vitro it is very active against avian PPLO, swine PPLO, and spirochetes. In animal tests it is very effective against three species of Leptospira, erysipelas of turkeys, avian PPLO, Hemophilus pertussis, Hemophilus influenzae, Moraxella bovis, one species of avian coccidia, and several intestinal parasites [7]. In either animal or tissue-culture tests it is very active against the large viruses causing infectious synovitis of chickens, meningopneumonitis of mice, and ornithosis.

METHODS

Experimental infections of chickens and turkeys were used to study the effect of tylosin against avian PPLO. In chicken-infection tests, three-week-old commercial broilers were injected with 0.2 ml of a pure culture (usually 10^6 or 10^7 organisms/ml) of PPLO by both the intravenous and intra air-sac routes. During a four-week period following treatment observations were made of average weight gains, the incidence and severity of air-sac lesions, blood antibodies to PPLO, and the isolation of PPLO, bacteria, and viruses [8]. Chickens were treated with tylosin by dust inhalation, addition to the drinking water, or injection.

For infectious sinusitis tests the sinuses of six-week-old poults were injected with a pure culture of PPLO and treatment administered after swelling had developed. Observations were made during a four-week period for the incidence and severity of sinus swelling and cultures made to isolate PPLO. Tylosin was administered to turkeys by direct injection into the sinuses.

RESULTS

The experimental PPLO infections in chickens varied in intensity from one test to another, apparently governed by the virulence of the particular PPLO strain and the susceptibility of the birds; however, the systems were basically reproducible. The infection caused marked depression of weight gains and high incidence of air-sac lesions. It was believed that the pathology observed was produced entirely by the injected PPLO since no viruses were found and bacteria were isolated from only a low percentage of chickens. PPLO were not isolated from noninfected chickens in most instances.

Sinusitis in turkeys was obvious within one week after inoculation and was developed for clinical evaluation within two weeks.

It will be seen in the following tables that adequate dosages of tylosin as the lactate or tartrate salt were highly effective in controlling the PPLO infections of chickens and turkeys. Generally, the dosages necessary in chickens were as follows: 5 mg/lb of body weight by injection, 2 g/gal of drinking water for a period of 3 to 5 days, or dust insufflation at the rate of 50 mg/ft^2 of floor space,

Table 1. Infection with PPLO Strain 295, Four-Week Summary

Treatment[*]	Method of administration	Average weight gain, g	PPLO, SP test	Lesions		PPLO isolation[§]
				Gross air-sac	Histological[‡]	
Noninfected		815	0/30[†]	0/30	1/15	0/23
Infected		628	28/29	24/29	12/16	13/25
Infected, tylosin 5 mg/lb	Intraperitoneal injection	880	18/30	1/30	5/15	3/23
Infected[‖], tylosin dust 50 mg/ft^2	Inhalation	780	25/30	2/30	5/15	3/24
Infected[#], tylosin 2 g/gal	Via drinking water	829	17/30	1/30	2/14	0/21

[*] Thirty chickens per treatment. Average initial weight 213 g.

[†] Number positive/number examined.

[‡] Includes lesions of trachea and/or air sacs of each chicken.

[§] Includes PPLO isolation from trachea and/or air sac.

[‖] Treatment administered three times at 24-hour intervals.

[#] Treatment initiated 3 days before and continued through 7 days after experimental infection.

three times at 24-hr intervals. Infectious sinusitis was controlled by doses of 1 mg/sinus.

Experiment 1. The chickens in this experiment were infected with PPLO strain 295 and treated immediately with tylosin by one of three routes (Table 1). The intraperitoneal (air sac) injection of tylosin was given on the opposite side from the PPLO inoculum. The tylosin-dust inhalant was administered at the rate of 50 mg/ft^2 of floor space on the day of infection and repeated two more times at 24-hr intervals. Tylosin tartrate was added to the drinking water at the rate of 2 g/gal, beginning 3 days before infection and continuing through 10 days. All tylosin treatments were highly effective in controlling the PPLO infection, as demonstrated by the relative average weight gain and the incidence of lesions and recovery of PPLO. It should be noted that the average weight gain of two of the groups exceeded that of the noninfected control group.

Experiment 2. After the chickens had been given an injection of PPLO culture in the usual manner, certain groups were treated with tylosin by one of three methods. The results shown in Table 2 illustrate the high degree of efficacy of the antibiotic when administered in sufficient dosage. The optimum dose in these experiments has appeared to be 5 mg/lb of body weight, administered parenterally. As is shown, a dosage of 2.5 g/gal of drinking water is more effective than one of 1.0 g/gal. The higher dose has been assumed to be a practical level to control the condition. In other studies it has been found that treatment for a period of 3 to 5 days is as effective as treatment for 7 days.

Table 2. Infection with PPLO Strain 699, Four-Week Summary

Treatment*	Average weight gain, g	PPLO, SP test	Lesions			PPLO isolation
			Gross		Histological incidence	
			Incidence	Index		
Noninfected	798	0/30†	0/30	0	4/15	0/21
Infected	557	26/26	26/26	3.5	14/14	14/21
Infected, tylosin 5 mg/lb	732	10/30	1/30	0.1	2/15	2/22
Infected, tylosin 2.5 g/gal 7 days	760	12/30	5/30	0.4	4/15	6/23
Infected, tylosin 1.0 g/gal 7 days	584	29/30	24/29	2.9	14/15	14/23

*Thirty chickens per treatment.
†Number positive/number examined.

Experiment 3. The purpose of this experiment was to compare tylosin treatment initiated at the time of infection to treatment initiated 5 or 10 days after infection (Table 3). When treatment was delayed more opportunity was allowed for the infection to become established. Differences were apparent when the incidence of lesions, agglutinins, and PPLO isolations were compared between the controls and the various treatments. This would be expected with almost any disease and treatment. It is interesting to note, however, that the weight gains were supported well in the groups where treatment was delayed. Some coccidiosis was present in the chickens and may account for the low rate of gain in the one tylosin-treated

Table 3: Infection with PPLO Strain 699, Drinking Water-Tylosin Lactate Treatment, Four-Week Summary

Treatment*	Average weight, g	PPLO, SP test	Lesions		PPLO isolation§
			Gross air sac	Histo-logical‡	
Noninfected	607	0/29†	0/29	1/14	0/20
Infected	536	30/30	29/30	16/16	18/20
Infected, tylosin 2.5 g/gal 3 days	566	11/30	9/30	6/15	3/20
Infected‖, tylosin 2.5 g/gal	626	29/30	21/30	14/15	8/20
Infected#, tylosin 2.5 g/gal	632	24/24	23/24	9/9	6/14

*Thirty chickens per treatment.
†Number positive/number examined.
‡Includes lesions of trachea and/or air sacs of each chicken.
§Includes PPLO isolation from trachea and/or air sac.
‖Treatment initiated 5 days after experimental infections and continued for 3 days.
#Treatment initiated 10 days after experimental infection and continued for 3 days.

Table 4. Efficacy of Intramuscular Administration of Tylosin in Eliminating PPLO from Tissues of Experimentally Infected Chickens

Treatment*	PPLO SP test		Air-sac lesions		PPLO isolated			
					Trachea		Air sac	
	7‡	14	7	14	7	14	7	14
None	10/10§	10/10	10/10	10/10	10/10	10/10	1/10	9/10
None	10/10	10/10	10/10	10/10	10/10	10/10	4/10	9/10
Tylosin† 25 mg/kg	8/10	9/10	5/10	1/10	0/10	1/10	1/10	0/10
Tylosin† 25 mg/kg	10/10	5/10	8/10	0/10	0/10	1/10	0/10	0/10

*Twenty chickens per treatment.
†Tylosin a administered intramuscularly for 3 consecutive days beginning 7 days after infection.
‡Days after treatment.
§Number positive/number examined.

group. At the end of the experiment, even with delayed treatment, the air sac lesions were healing in the tylosin-treated groups and were not as severe as in the infected control group.

Table 5. Turkey Sinusitis Induced with PPLO Strain 540 and Treated by Sinus Injection of Tylosin, Four-Week Summary

Treatment[*]	Time, weeks	Swollen sinuses	Percent swollen	Index of swelling[‡]
Infected	0	14/15[†]	93.3	2.5
	2	12/15	80.0	2.8
	4	13/15	86.7	3.0
Infected,	0	14/15	93.3	2.5
tylosin	2	2/15	13.3	1.1
25 mg/sinus	4	0/15	0.0	1.0
Infected,	0	14/15	93.3	2.5
tylosin	2	1/15	6.7	1.1
10 mg/sinus	4	0/15	0.0	1.0

[*] Fifteen turkeys per treatment.

[†] Number swollen/number examined.

[‡] Key to index: 1 = nonswollen, 2 = mild swelling, 3 = moderate swelling, 4 = marked swelling.

Experiment 4. A study was designed to determine the action of tylosin in eliminating Mycoplasma gallinarum from a measured amount of tracheal and air-sac tissues of experimentally infected chickens. PPLO infection was by the usual routes and two groups were treated with tylosin on 3 consecutive days, beginning 7 days after infection (Table 4).

Of 20 chickens from the initial group studied at necropsy prior to the experiment, none had air sac lesions, serum agglutinins for PPLO by the serum plate agglutination (SP) test, or possessed serological evidence of the presence of common avian respiratory viruses.

As seen in Table 4, the incidence of air-sac lesions and reactions to the PPLO SP test indicates that all chickens were infected. The number of chickens

Table 6. Turkey Sinusitis Induced with PPLO Strain 540 and Treated by Sinus Injection of Tylosin, Four-Week Summary

Treatment[*]	Time, weeks	Swollen sinuses	Percent swollen	Index of swelling[‡]
Infected	0	13/18[†]	72.3	2.5
	2	17/17[§]	100.0	3.2
	4	15/15[‖]	100.0	4.0
Infected,	0	13/18	72.3	2.5
tylosin	2	3/15[#]	20.0	1.3
4 mg/sinus	4	1/15	6.6	1.1
Infected,	0	13/18	72.3	2.5
tylosin	2	2/18	13.3	1.1
1 mg/sinus	4	0/18	0.0	1.0

[*] Eighteen turkeys per treatment.

[†] Number swollen/number examined.

[‡] Key to index: 1 = nonswollen, 2 = mild swelling, 3 = moderate swelling, 4 = marked swelling.

[§] One turkey died.

[‖] Two turkeys died.

[#] Three turkeys died.

positive to the SP test was reduced in one group following tylosin treatment. The titers of serum agglutinins following therapy were approximately one log dilution lower than the corresponding nontreated controls. There was a remarkable difference in the incidence of isolation of PPLO between the treated and nontreated groups, particularly from tracheal tissues. It may be noted that the incidence of air-sac lesions had almost disappeared by 14 days after treatment, in contrast to the nontreated chickens. Although three PPLO isolations were made out of 80 attempts, it appears possible to completely eliminate the organisms from these tissues with tylosin therapy.

Experiment 5. In this experiment turkeys were infected by intrasinus injection of pure culture PPLO when they were six weeks of age and treated with tylosin tartrate two weeks later. This summary is an accumulation of the four-week observation period after treatment (Table 5). The reduction in the numbers of swollen sinuses in the tylosin-treated groups is obvious. Also note the increase of the index of sinus swelling in the nontreated controls, which indicate that the condition had increased in severity. Ten milligrams of tylosin/sinus were as effective as 25 mg/sinus.

Experiment 6. This experiment was conducted in the same manner as the preceding one. Notice that the dosages of tylosin were either 4 or 1 mg/sinus and that the results were very similar to those obtained with higher dosages (Table 6). The number of swollen sinuses and the severity of swelling increased during the experiment in the nontreated control group. In all experiments of this type, even with low dosages of tylosin, obvious improvement of the sinus swelling was apparent within one week and swelling was completely reduced in nearly all turkeys within two weeks after treatment.

DISCUSSION

In the tylosin project at the Lilly Agricultural Research Center, more than 40 experiments have been completed using experimental PPLO infections of chickens and turkeys. Six typical experiments have been reviewed in this paper. The studies have demonstrated that tylosin is highly effective in the control of PPLO infection of chickens and turkeys. They also show that a virulent strain of avian PPLO can markedly influence the health of chickens and drastically reduce weight gains and carcass quality without the influence of other infections. In naturally occurring chronic respiratory disease PPLO are the primary cause but the picture is frequently complicated by secondary bacterial invaders. This evidence indicates that tylosin has superior activity against avian PPLO and, since these PPLO are the primary agents of chronic respiratory disease, their control or elimination may result in a more satisfactory treatment. It also suggests the use of tylosin at the time of vaccination or earlier to reduce or prevent PPLO infection at the time of stress. One experiment strongly suggests that tylosin may completely eliminate PPLO with adequate dosage. Field studies with tylosin treatment of naturally occurring cases of CRD have essentially substantiated these experimental findings.

SUMMARY

Experimental evidence showed that tylosin was highly effective in the control of virulent pleuropneumonia-like-organism infections of broilers at dosages of 5 mg/lb of body weight by injection, 2 g/gal of drinking water for 3 to 5 days, or 50 mg of tylosin dust/ft^2 of floor space, three times at 24-hr intervals. Tylosin

has been highly effective in control of experimental PPLO sinusitis of turkeys in dosages as low as 1 mg/sinus.

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DISCUSSION

Q: Number one, Dr. Barnes, have you done any work with day-old chickens or young chicks under three weeks? Number two, have you compared tylosin with other antibiotics such as erythromycin or chlortetracycline?

DR. BARNES: We have not worked with day-old chicks in our experimental system. We had some work under field conditions with young chicks and this was a 5000-bird operation as I recall it. These chicks were treated, and at the time of vaccination, were four weeks of age. A break occurred in that flock following vaccination and there was a very decided difference in improvement in the birds that had been treated with tylosin. I do not recall the data but the break was very severe in the untreated birds. Comparisons with other antibiotics have been performed but, with the possible exception of streptomycin, we showed superior activity with tylosin.

Q: Would you care to comment on the chemistry of this material and, in view of the low toxicity reported, would there be any extension to this for humans. If so, what are the data?

DR. BARNES: Tylosin is a macrolid type of antibiotic, with a large ring structure, and in certain of its antibiotic activities it resembles erythromycin. However, we feel that it shows superior activities to erythromycin in terms of the PPLO, spirochetes, and large viruses, particularly the ornithosis group of agents. Extension into human medicine is under consideration.

MICROORGANISM CONTROL IN CANE SUGAR MILLS

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INTRODUCTION

Normal cane juice appears to be an almost ideal medium for the growth of a wide variety of microorganisms. It contains approximately 14% sucrose, adequate amounts of nitrogenous compounds, and a number of growth factors. It has a favorable pH and temperature when it is freshly expressed from the cane. Certain procedures in harvesting, such as burning the leaves from the cane, greatly increase the initial number of microorganisms present in the expressed juice. Owen [1] states that if a mill is operating under highly sanitary conditions the juice from the last mill will show little evidence of deterioration in 5 hr, based on sucrose content. If we use this criterion as the index of sanitation, then few indeed are the mills which operate under highly sanitary conditions.

As the juice splashes over the interior of mill housings, flows through troughs and pipes during recirculation, and then flows to the heaters and clarifiers, it comes into direct contact with countless numbers of microorganisms attached to metal and concrete surfaces. Together with dirt and very small cane fibers these microorganisms form a slime layer or accretion which can readily be observed on the lower part of the interior of the mill housings, in the troughs which discharge juice over the cane entering the various mills, and wherever metal or concrete surfaces over which the juice passes are exposed. Microscopic examination of this slime accretion reveals the presence of many bacteria, molds, and yeasts, some dead and many living. When a portion of such a slime is added to freshly expressed cane juice of known purity and known reducing-sugar content, a rapid decrease in purity will be accompanied by a more rapid increase in glucose ratio. This change is often measurable within 10 min and is quite pronounced within 30 min and is, therefore, attributed to enzymatic action rather than to the direct growth of bacteria and yeasts in the juice. This process is known as microbial inversion and the enzyme, as invertase.

Ordinary yeast, several types of molds, and many types of bacteria secrete invertase. Very minute amounts of invertase added to sucrose solutions will rapidly invert a part of the sucrose into glucose and fructose. In addition to the invertase secreted by microorganisms there may be significant amounts of it in the juice at various times as it is expressed from the cane. The slime accretion found on the various parts of the mill equipment not only contains bacteria, molds, and yeasts but it is in constant contact with freshly expressed and recirculating juice, to which it adds both microorganisms and the enzymes they secrete. This slime is unquestionably the most important source of invertase in cane juice.

While the need for microorganism control during the grinding of cane should be obvious to a microbiologist, it is not generally so to many sugar mill technicians. When large quantities of translucent cohesive pellets collect below coarse juice screens, settle out in juice tanks, and later impede the flow from tanks into pipes and through pumps, then mill technicians recognize a serious infection of dextran-producing microorganisms. The trained microbiologist will readily recognize this infection as one of Leuconostoc mesenteroides. This infection must be controlled quickly in order to avoid large losses in sucrose, not only by actual destruction by the bacteria and their enzymes, but also from huge losses of nonprocessable cane juice. The writer knows of one such stoppage due to Leuconostoc slime when 25,000 gal of juice containing 11 tons of sucrose had to be run into the sewer because it was in a semisolid condition due to its high dextran content. While such infections do not occur in every sugar mill, they are sufficiently known in most cane areas to be a problem of general interest. Another recognizable effect of a lack of good microorganism control is a sour odor due to acid fermentations. A common treatment in this case is the use of slaked lime, which obviously does not get at the cause of the trouble. The most general need for microorganism control is reflected in the loss of sucrose content in the mixed juice which goes to the factory as compared with the sucrose content of the original crusher juice from the cane. A reliable index of this loss is obtained by comparing the glucose ratio of the crusher juice with the glucose ratio of the mixed juice. By definition, the glucose ratio = percent reducing sugars × 100/percent sucrose (apparent). Any increase in the value of this ratio represents a loss in sucrose of a definite magnitude. While it is not expected that all of this loss will be prevented by good microorganism control during the grinding, increases in yield of 1 to nearly 4 lb of commercial sugar/ton of cane ground have been obtained during the application of an effective bactericide. The significance of this improved yield can be readily seen when one realizes that an average sized mill grinds some 3,000 tons of cane every 24 hr. In such a case the increased yield of commercial sugar through microorganism control could be between 1.5 and 6 tons/24 hr.

CONTROL OF MICROORGANISMS DURING THE GRINDING OF CANE

Good housekeeping is the first important means of microorganism control and no microbiological control can be adequate without regular and thorough cleanups. Frequent and thorough cleaning of mill equipment with hot water and steam removes much dirt and fine fibers from portions of the equipment which are accessible during grinding. Some of the slime accretion is also removed but much of it cannot be removed in this way. To the extent that the slime accretion is not removed, the practice of daily or weekly cleanups is an inadequate means of microorganism control.

The inactivation of invertase either by chlorine or chlorine-releasing compounds is being used as one means of reducing sucrose losses during the grinding of the sugar cane. Since chlorine reacts quickly with all types of organic matter in the juice and with the bagasse itself, its application is necessarily limited to short periods of contact between the juice and the chlorine, during which time some invertase is inactivated. In addition to the corrosive action of chlorine gas and chlorine liberated from hypochlorities, there is inadequate control of microorganisms in the slime accretion not directly contacted by the chlorine. As stated previously, slime accretion on mill equipment constitutes the most important source of invertase and unless such accretion is eliminated it will continue to secrete invertase in those parts of the mill not treated with chlorine or chlorine-releasing compounds.

An entirely new means of microorganism control in cane sugar mills consists of applying a new organic-sulfur type of bactericide. This product, known as bactericide A,* is added at carefully selected points in the tandem to assure the treatment of both juice and mill equipment. This bactericide is introduced into the juice or sprayed over the cane entering a particular mill so that in effect the bactericide is carried by the juice to all parts of the grinding equipment, troughs, and pipes contacted by the juice. It is possible to introduce bactericide A in this manner because its activity is not affected either by the organic matter in the juice or by the bagasse. Since juice with bactericide continuously flows over surfaces and through troughs, every part of the grinding surface contacted by the juice will be contacted by the bactericide.

ELIMINATION OF BACTERICIDE FROM RAW SUGAR AND MOLASSES

On the basis of the observed behavior of dilute solutions of bactericide A and similar combinations of organic sulfur compounds, it is known that the conditions of pH and temperature to which cane juice is normally subjected during clarification and evaporation result in the decomposition of the disodium cyanodithioimidocarbonate and the potassium N-methyldithiocarbamate. In our laboratories we have obtained direct experimental evidence concerning the decomposition of these two compounds from the study of their ultraviolet and infrared absorption spectra.

Freshly diluted solutions of bactericide A were heated at 60, 80, and 100 C for 15 min prior to the determination of their ultraviolet spectra. When these spectra were compared with the ultraviolet spectrum of an unheated, freshly diluted solution of this product [2], it was evident that nearly complete decomposition of the original organic sulfur compounds had occurred at 80 C and that complete decomposition had occurred at 100 C. These conditions of time and temperature are comparable to those used in the clarification and evaporation of cane juice. Furthermore, dilute solutions of bactericide A slowly decompose upon standing at room temperature, as shown by their ultraviolet spectra after a period of six hr.

Solutions of disodium cyanodithioimidocarbonate decompose below pH 7 and yield carbon disulfide, identified by its infrared spectrum. Since carbon disulfide has been split from this molecule, cyanamide remains in the solution and undergoes its usual reactions which lead to urea and related compounds.

Dilute potassium N-methyldithiocarbamate solutions at a pH below 7 yield hydrogen sulfide and sulfur. More concentrated solutions also yield methyl isothiocyanate. The hydrogen sulfide and methyl isothiocyanate are volatile and in the evaporation of cane juice they would be removed by a steam distillation process. Sulfur would be removed during the clarification process, which involves the use of lime. This removal of sulfur has been confirmed at Louisiana State University by the analyses of sludges during pilot-plant experiments with bactericide A added to cane juice.

In addition to the evidence of degradation of Bactericide A, it has been found that whenever this product is used in very dilute concentrations in any water-process system it is necessary to add it either continuously or over an extended period of time in order to achieve effectiveness against microorganisms. All of

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*Bactericide A is a commercial product, Busan 881, manufactured by Buckman Laboratories, Inc., Memphis, Tennessee. It has the following composition:

Active ingredients	
Disodium cyanodithioimidocarbonate	12.7%
Ethylenediamine	4.8%
Potassium N-methyldithiocarbamate	17.5%
Inert ingredients	65.0%

the lines of evidence presented support the conclusion that bactericide A does not contribute residues to either raw sugar or blackstrap molasses.

APPLICATION OF BUSAN 881 IN VARIOUS MILLS

During the 1957 sugar campaign in Cuba, a new organic-sulfur type of bactericide was introduced [3] to reduce the inversion of sucrose in cane juice prior to its clarification. This work continued into the 1958 campaign with the application of bactericide A [2], with the result that this product became recognized as useful in microorganism control in cane sugar mills. In 1958 one of the large Mexican sugar mills began to use this product and continued to use it during the 1959 campaign, when the marked effectiveness of this product in controlling the dextran-producing *L. mesenteroides* was demonstrated clearly. One large Brazilian sugar mill and one large Peruvian sugar mill used this same product successfully during the 1959 campaign. During the 1960 campaign in Argentina, eight sugar mills used this product and two of these had severe Leuconostoc infections which were successfully controlled.

To illustrate the method employed by the writer to calculate the sucrose savings realized by the application of bactericide A in a particular mill, some of the data from one large Brazilian sugar mill will be discussed briefly. This mill grinds approximately 3,500 metric tons of cane daily, from which approximately 350 tons of commercial sugar are extracted. Data from the mill records are given in Table 1 for the period September 6 to October 3, 1959. During the period from September 13 to September 22, Bactericide A was added continuously to the water of imbibition at the rate of 15 ppm, based on the weight of the cane. The weeks from September 6 to 12 and from September 27 to October 3 represent conditions immediately before and after treatment.

Table 1. Variation of Drop in Purity and Increase in Glucose Ratio Between Crusher and Mixed Juices

Week of grinding, 1959	Brix	Apparent sucrose	Purity	Invert sugar	Glucose ratio	Drop in purity	Increase in glucose ratio
Sept. 6-12			No bactericide added				
Crusher juice	21.78	19.08	87.60	0.82	4.29		
Mixed juice	18.03	15.12	83.86	1.01	6.68	3.74	2.39
Sept. 13-19			15 ppm of Bactericide A added				
Crusher juice	21.99	19.27	87.63	0.82	4.25		
Mixed juice	18.30	15.64	85.46	0.84	5.37	2.17	1.12
Sept. 20-26		15 ppm of Bactericide A added Sept. 20-22					
Crusher juice	22.42	19.09	85.18	1.10	5.76		
Mixed juice	18.54	15.40	83.06	1.16	7.53	2.12	1.77
Sept. 27-Oct. 3			No bactericide added				
Crusher juice	22.56	18.98	84.13	1.40	7.38		
Mixed juice	19.44	15.89	81.74	1.52	9.56	2.39	2.18

For any given week of grinding in any sugar mill the average purity of the crusher juice is always larger than that of the mixed juice. On the other hand the average glucose ratio of the crusher juice is always smaller than that of the mixed juice. Both of these changes indicate the same thing, namely, a loss in sucrose content during the period when the juice passes through the grinding process. If the drop in purity and the increase in glucose ratio from the crusher juice to the mixed juice during the first week (Table 1) are compared with the corresponding values for the second week it is evident that smaller changes occurred during the second week. The reverse order of changes will be noted when the third week is compared with the fourth week. Since these changes in the drop in purity and in the increase in glucose ratio are directly related to sucrose content, it is evident that the sucrose losses are much smaller in the second and third weeks than in the first and fourth weeks. Smaller losses of sucrose mean savings and these result from application of the bactericide. The magnitude of these sucrose savings can be readily calculated from either the drop in purity data in Table 1 or from the increase-in-glucose-ratio data. The details for these calculations will be found elsewhere [4].

Using the increase-in-glucose-ratio data in Table 1 for the first and second weeks, it has been calculated that the sucrose savings expressed in weight of commercial sugar were 1.89 kg/metric ton of cane ground or 6.6 metric tons of commercial sugar/day. Approximately the same results were obtained by using the drop in purity data for these two weeks. This savings can be expressed in another way; 6.6 tons of sugar represents a 1.9% increase in the daily sugar production. The value of the savings greatly exceeds the cost of the bactericide applied.

Another example of good sugar savings from the application of 15 ppm of bactericide A has been reported [4] for a large sugar mill in Peru which grinds approximately 3,200 metric tons of cane daily during an 11-month campaign. In this mill the calculated sugar savings were 0.89 kg of commercial sugar/metric ton of cane ground or 2.9 metric tons of commercial sugar/day. This represents an increase of 0.9% in the daily production of sugar.

A different example of benefits will be cited for a large mill in Mexico. Due to large and unpredictable variations in the quality of the cane supplied to this mill, no reliable weekly averages for the sugar contents of the crusher and mixed juices were available to calculate the sugar savings. Bactericide A was introduced in this mill for the purpose of eliminating slime accretions responsible for producing much inversion of sucrose and for obstructing screens and juice lines. The dosage applied varied between 15 and 20 ppm based on the weight of the cane ground. An entirely satisfactory slime-control program, including the elimination of Leuconostoc slime, continued throughout the 1959 grinding season.

SUMMARY

A satisfactory degree of microorganism control during the grinding of sugar cane has been demonstrated repeatedly in various sugar mills of the Western Hemisphere. Either one or both of two criteria have been employed to demonstrate the benefits derived: (a) elimination of dextran-producing bacteria such as Leuconostoc mesenteroides, (b) improvement in sugar recovery to the extent of approximately 1 to 2%, based on the daily production. The bactericide employed in this work was an organic-sulfur type containing the active ingredients, disodium cyanodithioimidocarbonate, potassium N-methyldithiocarbamate, and ethylenediamine. Extensive analytical work has been completed to determine whether or not any detectable residue from this product remains in the raw sugar and final molasses produced from juice to which 10 to 20 ppm of this bactericide have been added. There is no detectable residue.

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SURVIVAL OF SALMONELLA ON WAXY FRUIT

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INTRODUCTION

Inquiries relative to the role of waxy fruit as a carrier of disease-producing species of Salmonella are common. However, a review of the literature provides no direct information upon which to base safe estimates of the potential hazard involved in eating such fruits as apples, pears, and grapes when they have been picked or handled by typhoid carriers or individuals carrying species of Salmonella other than Salmonella typhosa.

Apples, pears, and grapes have not been incriminated on an epidemiological basis as carriers of diseases of this type. Nevertheless, advertising literature and labeling for detergent-sanitizers and germicidal rinses are occasionally encountered which recommend these products for decontaminating market fruit. Such literature usually cites the potential danger to the consumer of fruit contaminated by filth and unhealthy handlers and includes direct references to Salmonella infections of one kind or another.

Some species of Salmonella, notably S. typhosa, die very rapidly during drying on inanimate surfaces. Others like Salmonella montevideo can survive for months or even years when dried on an inanimate object. However, the skin of a ripe waxy fruit is neither completely dry nor inanimate but is a living tissue actively engaged in respiration and other physiological activities.

Many germicidal chemicals are known to be effective against various species of Salmonella carried by farm building fixtures, food processing equipment, dairy utensils, dishes, and glassware. But the skin of living fruit cannot be classified with any of these inanimate materials and no safe estimates on the effectiveness of the germicides can be made. This study was designed, therefore, to develop in an exploratory manner direct experimental evidence for future use in answering inquiries on the role of waxy fruits as carriers of species of Salmonella of significance to public health and for evaluating effectiveness claims for various germicidal detergents recommended for decontaminating market fruit.

PROCEDURE

Cultures of two species, S. typhosa, ATCC 6539 and S. montevideo (strain furnished by W. R. North, Microbiological Division, Food and Drug Administration), were selected as test organisms based upon prior knowledge of resistance in drying. The former tends to die very rapidly during drying, death on inanimate surfaces usually occurring within 4-6 hr. The latter has frequently been encountered in dried materials after storage for three years. There seems to be reason to believe that these two cultures represented the minimum and maximum resistance levels for species within the rather heterogenic genus Salmonella insofar as drying was concerned.

Sound market fruit was obtained from local retail produce markets. Separate studies were made with Red Delicious, Summer Rambo, Golden Delicious, and

McIntosh Red apples, Bartlett and Bosc pears, and Red Malaga and Almeria grapes. Pooled samples of rotten apple pulp, prepared from parings taken from a large number of rotting apples of different varities and mixed in a Waring blender, were also employed.

The test cultures were grown in A.O.A.C. nutrient broth [1] with three consecutive 24-hr transfers and incubated at 37 C. All sound fruit was inoculated with individual sterile cotton swabs dipped in the 24-hr broth test culture. In each instance the swab was applied in a manner calculated to give a uniform distribution of the test organism over the entire surface. The artificially contaminated fruit was held in the laboratory safety hoods at normal room temperatures (23-28 C) and relative humidities (40-60%) for 48 hr. At 1, 6, 18, 24, and 48 hr a uniform sample of the apple or pear surface was taken using a flamed, sharp, metal cork borer to remove a plug about 0.5 in. deep. This plug was transferred aseptically to tubes containing either 10 ml of sterile tetrathionate broth base [2] and/or trypticase soy broth [3]. In each instance the tubes were shaken vigorously and aliquots were removed for dilution plate counting. Tryptone-glucose extract agar [4] was employed in making all plate counts. The remaining portion of the tetrathionate-broth-base or trypticase-soy-broth fruit-plug sample was incubated for 24 or 48 hr at 37 C, depending on the absence or presence of visible growth, and streaks were made on individual plates of Salmonella-Shigella agar (S.S. Agar) [5] for quantitive recovery by enrichment procedures. These plates were incubated at 37 C for 24 hr.

With grapes, the fruit-surface sampling procedure varied in that one half of a single grape was found to provide a surface sample of approximately the same area as the cork-borer plug. Thus sampling consisted of cutting individual grapes in half with a sterile scalpel and implanting them in the subculture broths.

With the rotten-apple-pulp samples, inoculations were made using 200-g samples and 5.0 ml of the 24-hr broth culture. The inoculation was made with a sterile 5-ml pipette and the inoculum was mixed with the pulp by whipping with a sterile glass rod. The inoculated samples were held in bacteriological safety hoods at room temperature under petri dish covers for 48 hr. One gram samples were withdrawn at the same intervals employed with the sound fruit and transferred to 99 ml of sterile water. After vigorous shaking, 1-ml aliquots of this dilution were employed for inoculating tubes of trypticase soy broth. This dilution was also employed as the primary dilution in dilution plate counting.

Whole apples of the various varieties indicated above were employed for determining the relative effectiveness of various detergents and germicidal rinses in decontaminating inoculated sound-fruit surfaces. *S. montevideo* was the test organism of choice. The results with this organism might be expected to provide a more accurate index to the activity of the chemicals on bacteria of this genus since the interfering factor of death due to drying would be smaller than with *S. typhosa* or most other species. Surface areas were sampled and cultured before and after application of the chemical solution under test in the same manner as that described for the holding studies. Tetrathionate-broth-base enrichment culturing was not employed.

A wide variety of chemical solutions was tested at 26 C. Each inoculated apple was immersed in approximately 400 ml of the treating solution and agitated for 0.5, 1, and 2 min. The short time intervals were selected since they appeared more practical in terms of applications possible in actual practice and seemed necessary to avoid injury to the fruit.

Sodium hypochlorite was used as a source of available chlorine in a variety of alkaline and acid solutions. Adjustments in pH were made with solutions of sodium hydroxide, phosphoric acid, and acetic acid. In each instance the concentration of available chlorine was checked chemically by thiosulfate titration.

RESULTS

The results on the holding studies with the sound fruit were approximately the same for each test organism with all varieties of the three fruits studied. They are summarized in Tables 1 and 2 and Fig. 1.

It appears that S. typhosa, even when present in sound fruit in excessive numbers, will die within 24 to 48 hr from drying or other causes (Table 1). With S. montevideo this is not the case. This organism can be recovered readily

Table 1. Recovery of Salmonella from Inoculated Stored Sound Fruit Using Trypticase Soy Broth and Salmonella-Shigella Agar

Fruit	S. typhosa holding times, hr							S. Montevideo holding times, hr						
	0	0.5	1	6	18	24	48	0	0.5	1	6	18	24	48
Apples														
Red Delicious	+	+	+	+	+	−	−	+	+	+	+	+	+	+
McIntosh Red	+	+	+	+	+	−	−	+	+	+	+	+	+	+
Golden Delicious	+	+	+	+	+	−	−	+	+	+	+	+	+	+
Summer Rambo	+	+	+	+	−	−	−	+	+	+	+	+	+	+
Pears														
Bartlett	+	+	+	+	+	+	−	+	+	+	+	+	+	+
Bosc	+	+	+	+	+	+	−	+	+	+	+	+	+	+
Grapes														
Almeria	+	+	+	+	+	−	−	+	+	+	+	+	+	+
Red Malaga	+	+	+	+	+	+	−	+	+	+	+	+	+	+

+Growth
-No growth

Table 2. Recovery of Salmonella typhosa from Inoculated Stored Sound Fruit Using Tetrathionate Broth Base and Salmonella-Shigella Agar

Fruit	Holding times						
	0 hr	0.5 hr	1 hr	6 hr	18 hr	24 hr	48 hr
Apples							
Red Delicious	+	−	−	−	−	−	−
McIntosh Red	+	−	−	−	−	−	−
Golden Delicious	+	−	−	−	−	−	−
Summer Rambo	+	−	−	−	−	−	−
Pears							
Bartlett	+	−	−	−	−	−	−
Bosc	+	−	−	−	−	−	−
Grapes							
Almeria	+	−	−	−	−	−	−
Red Malaga	+	−	−	−	−	−	−

+Growth
-No growth

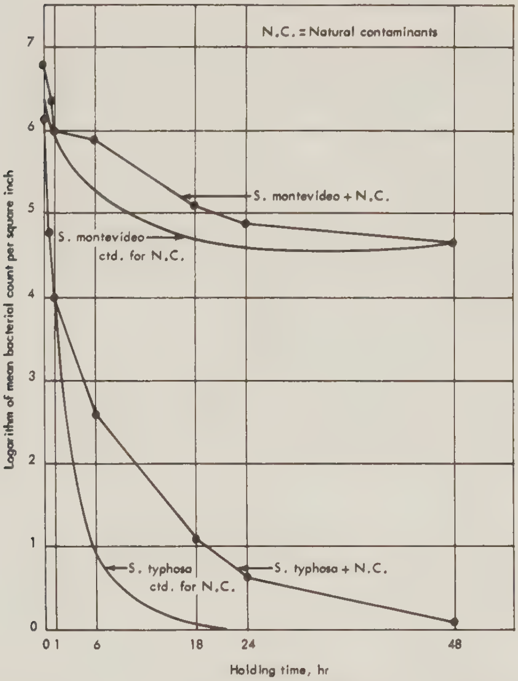

Fig. 1. Log survival curves for S. typhosa and S. montevideo developed from mean counts/in.^{2} of all sound fruit surfaces.

even after 48 hr. The trypticase-soy-broth incubation followed by streaking on Salmonella-Shigella agar gave uniform and reproducible results. However, with S. typhosa the tetrathionate-broth-base incubations when streaked on Salmonella-Shigella agar failed to give recovery of the test organisms when the inoculated fruit was held for even 30 min. The results with this organism (Table 2) indicate that the effects of drying for even 30 min on the individual cells of S. typhosa may be sufficient to make them susceptible to killing by the tetrathionate ion in this selective enrichment medium. These results would seem to rule out the tetrathionate broth base as a selective enrichment medium for the isolation of Salmonella from fruit. The results in Fig. 1 clearly indicate that the survival of S. montevideo is of a much different order than that of S. typhosa. The actual total-count data is complicated somewhat by differences in the number of natural contaminants which were found on the individual fruits. These varied from a few colonies in some instances to a few hundred/in.^{2} Corrections made on the basis of the apparent magnitude of the natural-contaminant count with each fruit provides survival curves for each organism which are more uniform and in better accord with the qualitative recovery data than the mean total-count curves. It should be understood that no reliable differentiation is possible with colonies on tryptone-glucose extract agar and that these corrections are based on estimations from counts on inoculated fruits as well as the over-all picture with individual inoculated fruits. With S. typhosa it appears that the total count actually is less than 10 organisms/in.^{2} within 6 hr. A few continue to persist for 18 to 24 hr (Table 1). With S. montevideo the mean number of survivors even after 48 hr is greater than 40,000/in.^{2} of fruit surface. This difference in behavior is of about the same magnitude as that observed for inanimate surfaces in earlier unpublished studies. Survival of a small number of S. typhosa cells appears to occur on the

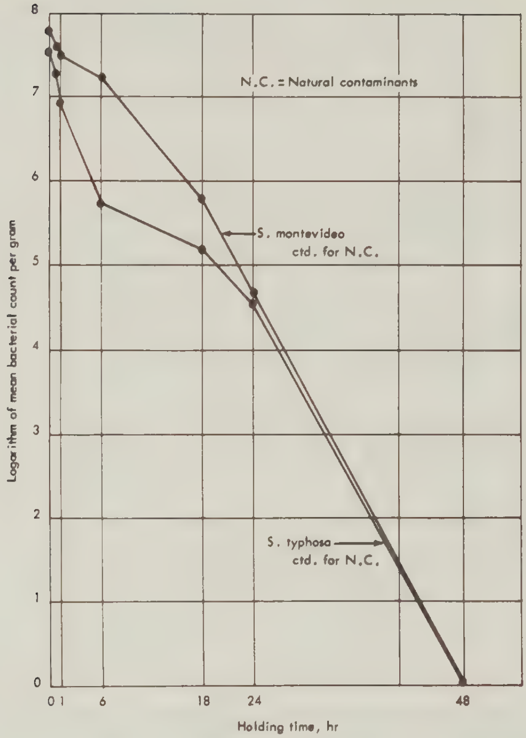

Fig. 2. Log survival curves for S. typhosa and S. montevideo developed from mean counts/g of rotten apple pulp samples.

surface of fruit longer than would be the case with an inanimate, completely dry surface. The results with the rotten-apple pulp samples are presented in Fig. 2 and Table 3.

Corrections for natural contaminants which formed colonies on the tryptone-glucose extract-agar dilution plates employed in counting were not as difficult to make with the rotten-apple-pulp samples as with the sound individual fruit samples since they were homogeneous mixtures and a total natural contaminant count could be made on uninoculated pulp. The results in Fig. 2 show that both S. montevideo and S. typhosa die in rotten-apple pulp. Complete death within 48 hr is indicated for both organisms by the survival counts shown in Fig. 2 and the enrichment culture isolation data listed in Table 3. The cause of death here is not known. The pH of the pulp was 4.2 and this in itself would not be expected to lead to the complete elimination of Salmonella. Obviously dehydration was not involved

Table 3. Recovery of Salmonella from Inoculated Stored Samples of Rotten Apple Pulp Using Trypticase Soy Broth and Salmonella-Shigella Agar

Organism	Holding times						
	0 hr	0.5 hr	1 hr	6 hr	18 hr	24 hr	48 hr
S. typhosa	+	+	+	+	+	+	−
S. montevideo	+	+	+	+	+	+	−

+Growth
-No growth

and the order of death can be seen to be different from that encountered on the surfaces of sound fruit, where dehydration appears to be a primary factor, by comparing the curves in Fig. 1 and 2. No real difference in the behavior of the two test species in rotten-apple pulp is indicated. It seems probable that antibiotic substances produced by the rot fungi may have played a major part in the results observed.

Results obtained in decontamination tests on inoculated sound whole fruit with detergents and various germicidal chemicals were uniformly negative insofar as complete elimination of *S. montevideo* was concerned. In many instances the count reductions were substantial and conclusions based on dilution-plate-count data alone would, in some instances, have indicated elimination of the test organism. However, incubation of surface-sample plugs in trypticase soy broth with subsequent streaking on Salmonella-Shigella agar showed clearly that small numbers of viable Salmonella survived the experimental treatments. The most effective material tested, in terms of count reduction, was sodium hypochlorite and some of the data obtained in tests on various preparations of this chemical are listed in Table 4.

The data listed in Table 4 clearly indicate that small residues of viable Salmonella may survive in sound market fruit washed in germicidal rinsing solutions normally accepted as effective in decontaminating precleaned inanimate surfaces. It should be acknowledged that the artificial contamination employed in these studies was heavy. Nevertheless the data clearly illustrate that Salmonella of the type which will survive drying on the surface of waxy fruit are not easily removed by dipping or washing procedures employing detergents and solutions of germicidal chemicals. Washing with solutions of commercial detergents and washing powders left residues of thousands of cells when the surfaces were inoculated and cultured in a similar manner.

If public health problems should develop involving waxy fruits and species of Salmonella, especially those resistant to drying, it would appear that an effective

Table 4. Data on Decontamination of Whole Sound Apples Inoculated with *S. montevideo* by Germicidal Rinsing Solutions Made with Sodium Hypochlorite

Available chlorine concentration, ppm	Solution pH	Chemicals used in pH adjustment	Immersion time at 26 C, min	Initial count/in.^{2} of inoculated apple surface. Tryptone-glucose extract agar	Average count after treatment/in.^{2} of apple surface. Tryptone-glucose extract agar	Trypticase-soy broth, Salmonella-Shigella isolation
200	10.5	NaOH	2	50,000,000	200	+
200	10.5	"	1	4,200,000	600	+
100	10.3	"	2	15,000,000	4000	+
100	10.3	"	1	2,200,000	2000	+
100	6.8	H_3PO_4	2	34,000,000	< 100	+
100	6.8	"	1	4,000,000	< 100	+
100	6.0	"	2	28,000,000	< 100	+
100	6.0	"	1	44,000,000	< 100	+
100	5.0	"	2	20,000,000	< 100	+
100	5.0	"	1	42,000,000	< 100	+
100	4.0	"	1	22,500,000	< 100	+
100	4.0	CH_3COOH	1	34,000,000	< 100	+
100	7.0	H_3PO_4	1	25,000,000	< 100	+

+
100 ppm alkyl (C_{14}, C_{16}, C_{12}) dimethyl, benzyl ammonium chloride

washing and rinsing process might have to be developed. Such a treatment would, in all probability, have to take cognizance of the waxy material present, its possible protective effect for the bacteria, and its role in the subsequent storage properties of the fruit itself. Such investigations are beyond the scope of these studies.

SUMMARY

Salmonella typhosa dies quite rapidly upon drying on the skin of sound waxy fruits such as apples, pears, and grapes. On apples and grapes complete death can be expected in 18 to 24 hr. On pears, the organism may survive for 24 but not 48 hr. The number of survivors is reduced to very low levels within 6 hr in all cases.

Salmonella montevideo does not die rapidly upon drying on the skin of apples, pears, and grapes. Large numbers of viable organisms may be found after a 48-hr drying or storage period. Both *S. typhosa* and *S. montevideo* are completely killed within 48 hr in rotten-apple pulp. The cause of death in rotten-apple pulp has not been determined.

If present, *S. montevideo* and probably other species of this genus resistant to drying cannot be completely removed from sound waxy fruits by washing or rinsing in germicidal solutions generally recognized as being effective in decontaminating inanimate surfaces. Such solutions may induce substantial reductions in the number of organisms present but a few viable cells will remain.

Waxy fruits are not known to be carriers of disease of the Salmonella type. It would appear that species causing some of these diseases would die between the time of contamination and consumption due to drying and/or other causes. Other species may persist on the skin of sound fruit and these cannot be removed completely by normal washing and rinsing methods developed for inanimate surfaces and employing germicidal chemicals.

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TETRACYCLINE SERUM CONCENTRATIONS IN HUMAN SUBJECTS FOLLOWING THE ORAL ADMINISTRATION OF AQUEOUS BUFFERED TETRACYCLINE SYRUPS

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INTRODUCTION

Since its introduction in 1953, numerous investigators have reported that tetracycline in various capsule formulations is well absorbed from the gastrointestinal tract [1-13]. Oil suspensions of this antibiotic were developed for pediatric use and in this form the antibiotic was only moderately absorbed from the gastrointestinal tract [15, 17-19]. A few brief studies have given data on the absorption of tetracycline from aqueous suspensions [14-16, 20]. Aqueous suspensions with various buffers have been developed to improve the absorption of tetracycline from the gastrointestinal tract. The results following the administration of various dosage regimens of these formulations are presented in this study.

METHODS AND MATERIALS

Two syrups were used in the clinical studies. One syrup contained 125 mg of tetracycline hydrochloride activity as tetracycline base and 125 mg sodium hexametaphosphate in each 5 ml. The second syrup contained 125 mg of tetracycline hydrochloride activity as tetracycline base and 125 mg ammonium polyphosphate in each 5 ml*. Both syrups contain suitable sweetening agents, preservatives, anti-oxidants, suspending agents, dispersing agents, flavors, and dyes. Both syrups are stable for at least two years, are quite palatable, and are easily administered to and readily accepted by children.

Regimens of a 5-ml and a 10-ml dose were used in the serum-concentration studies for each of the two syrups. Multiple dosage regimens were also used, 5 ml every 6 hr and 10 ml every 12 hr, for each of the two syrups.

The patients were normal young volunteers who had not been on antibiotic therapy for an adequate period of time previous to these studies. All medication was given in the fasting state. The medication was given by measuring exactly in a hypodermic syringe 5 or 10 ml and ejecting the contents directly into the mouths of the volunteers.

Pretreatment blood specimens were taken before administration of the medication and blood specimens were withdrawn at 1, 2, 3, 6, 8, 9, 12, 15, 18, 24, 36, 39, and 48 hr after administration.

Tetracycline assays were performed by the cup-plate method, employing Bacillus cereus var. mycoides as the test organism. All sera, including the pretreatment samples, were diluted at least threefold to eliminate spurious assays. Dilution was made with primary potassium phosphate buffer, giving a final pH of 4.5.

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*The trademark for Bristol Laboratories, a division of Bristol-Myers Company, for the aqueous syrup of tetracycline buffered with ammonium polyphosphate is Tetrex Syrup in the U.S.A. The trademark for Bristol Laboratories in foreign countries for the aqueous syrup of tetracycline buffered with sodium hexametaphosphate is Tetrex Syrup or Bristaciclina A.

RESULTS

Table 1 shows the number of patients and the average tetracycline serum concentration following the oral administration of 5 and 10 ml of the aqueous tetracycline syrup buffered with ammonium polyphosphate. The results are shown in Fig. 1.

Table 1. Tetracycline Serum Concentration in Human Subjects, Aqueous Tetracycline Syrup Buffered with Ammonium Polyphosphate

Time, hr	Number of patients	Average serum concentration*	Range	Number of patients	Average serum concentration†	Range
1	115	1.11	0-3.24	30	1.47	0-2.43
2	50	1.50	0.60-3.40	30	2.13	0.70-4.80
3	115	1.47	0-3.80	10	1.82	0.70-3.80
6	10	0.95	0.70-2.00	10	1.24	0.70-2.60
8	10	0.73	0.09-1.56	—	—	—
9	—	—	—	10	1.00	0.60-2.20
12	—	—	—	10	0.77	0.30-1.80
24	25	0.15	0-0.36	—	—	—

*µg tetracycline/ml plasma. Dose — 5 ml (125 mg) of tetracycline hydrochloride activity as tetracycline base buffered with ammonium polyphosphate.

†µg tetracycline/ml plasma. Dose — 10 ml (250 mg) of tetracycline hydrochloride activity as tetracycline base buffered with ammonium polyphosphate.

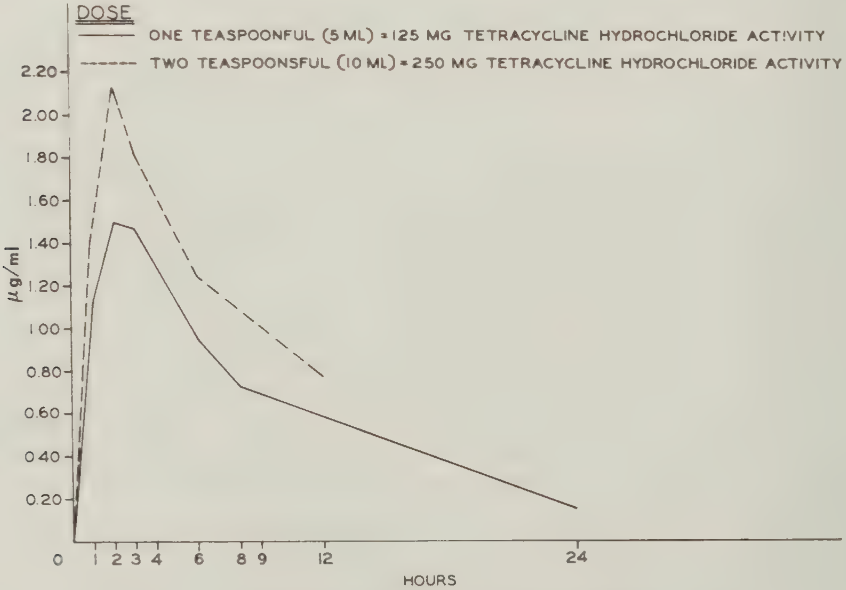

Fig. 1. Tetracycline serum concentration in human subjects, aqueous tetracycline syrup buffered with ammonium polyphosphate.

Table 2 shows the number of patients and the average tetracycline serum concentration following the oral administration of 5 ml every 6 hr and 10 ml every 12 hr of the aqueous tetracycline syrup buffered with ammonium polyphosphate. The results are shown in Fig. 2.

Table 2. Tetracycline Serum Concentration in Human Subjects, Aqueous Tetracycline Syrup Buffered with Ammonium Polyphosphate

Time, hr	Number of patients	Average serum concentration*	Range	Number of patients	Average serum concentration†	Range
1	10	1.05	0.80-1.40	10	1.03	0-2.00
2	10	1.46	0.90-2.40	10	1.89	0.70-4.80
3	10	1.30	0.80-2.50	10	1.82	0.70-3.80
6	10	0.95	0.60-2.00	10	1.24	0.70-2.60
9	10	1.47	0.80-2.90	10	1.00	0.40-2.20
12	10	1.06	0.50-2.30	10	0.77	0.30-1.80
15	10	1.83	0.90-3.50	10	2.09	1.10-4.60
18	10	1.37	0.70-1.60	10	1.58	1.00-3.60
24	10	1.80	1.10-3.30	10	1.05	0.60-2.30
36	10	2.11	1.30-3.50	10	1.45	1.00-2.40
39	10	2.48	1.50-4.10	10	2.91	1.50-4.60
48	10	2.27	1.40-4.10	10	1.52	0.90-2.70

*μg tetracycline/ml plasma. Dose – 5 ml (125 mg) every 6 hr of tetracycline hydrochloride activity as tetracycline base buffered with ammonium polyphosphate.

†μg tetracycline/ml plasma. Dose – 10 ml (250 mg) every 12 hr of tetracycline hydrochloride activity as tetracycline base buffered with ammonium polyphosphate.

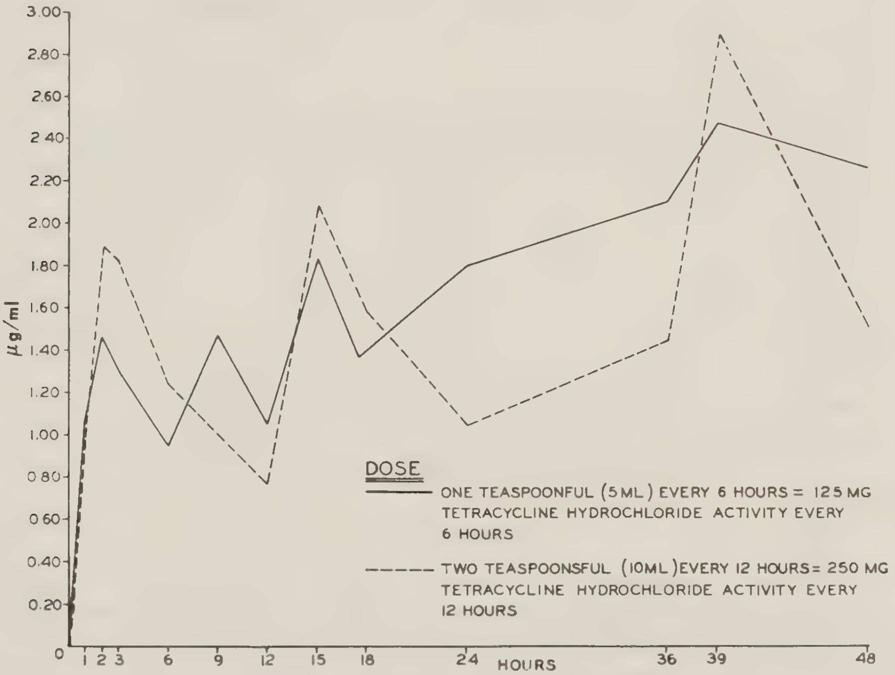

Fig. 2. Tetracycline serum concentration in human subjects, aqueous tetracycline syrup buffered with ammonium polyphosphate.

Table 3 shows the number of patients and the average tetracycline serum concentration following the oral administration of 5 and 10 ml of the aqueous tetracycline syrup buffered with sodium hexametaphosphate. The results are shown in Fig. 3.

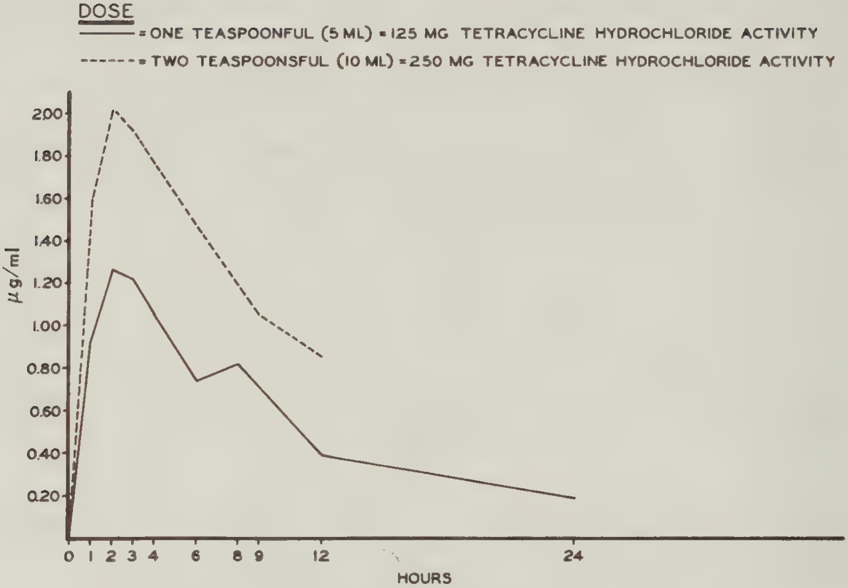

Fig. 3. Tetracycline serum concentration in human subjects, aqueous tetracycline syrup buffered with sodium hexametaphosphate.

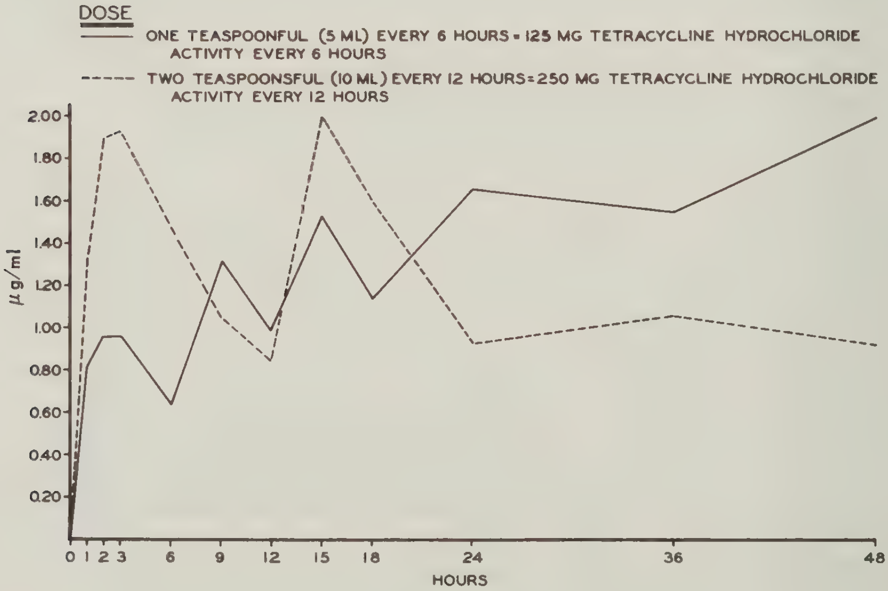

Fig. 4. Tetracycline serum concentration in human subjects, aqueous tetracycline syrup buffered with sodium hexametaphosphate.

Table 4 shows the number of patients and the average tetracycline serum concentration following the oral administration of 5 ml every 6 hr and 10 ml every 12 hr of the aqueous tetracycline syrup buffered with sodium hexametaphosphate. The results are shown in Fig. 4.

Table 3. Tetracycline Serum Concentration in Human Subjects, Aqueous Tetracycline Syrup Buffered with Sodium Hexametaphosphate

Time, hr	Number of patients	Average serum concentration*	Range	Number of patients	Average serum concentration†	Range
1	217	0.92	0-2.04	40	1.56	0.40-3.20
2	118	1.27	0-3.30	40	2.02	0.50-3.51
3	216	1.22	0-4.20	40	1.92	0.60-3.50
6	52	0.74	0.20-1.40	20	1.47	0.40-3.10
8	30	0.82	0-2.90	–	–	–
9	–	–	–	20	1.05	0.30-2.40
12	40	0.39	0-0.70	20	0.85	0.20-1.80
24	30	0.19	0-1.00	–	–	–

*μg tetracycline/ml· plasma. Dose – 5 ml (125 mg) of tetracycline hydrochloride activity as tetracycline base buffered with sodium hexametaphosphate.

†μg tetracycline/ml plasma. Dose – 10 ml (250 mg) of tetracycline hydrochloride activity as tetracycline base buffered with sodium hexametaphosphate.

Table 4. Tetracycline Serum Concentration in Human Subjects, Aqueous Tetracycline Syrup Buffered with Sodium Hexametaphosphate

Time, hr	Number of patients	Average serum concentration*	Range	Number of patients	Average serum concentration†	Range
1	20	0.81	0.20-1.40	20	1.33	0.40-2.20
2	20	0.96	0.40-1.60	20	1.90	0.50-3.20
3	20	0.96	0.30-1.70	20	1.93	0.60-3.50
6	20	0.64	0.20-1.20	20	1.47	0.40-3.10
9	20	1.32	0.60-2.50	20	1.05	0.30-2.40
12	20	0.99	0.50-1.80	20	0.85	0.20-1.60
15	20	1.53	0.90-2.50	20	2.00	1.00-3.50
18	20	1.14	0.70-1.80	20	1.60	0.70-2.70
24	20	1.65	1.00-2.30	20	0.93	0.40-1.50
36	20	1.55	0.50-3.60	20	1.06	0.50-2.90
48	20	1.99	0.50-3.40	20	0.92	0.60-2.00

*μg tetracycline/ml plasma. Dose – 5 ml (125 mg) every 6 hr of tetracycline hydrochloride activity as tetracycline base buffered with sodium hexametaphosphate.

†μg tetracycline/ml plasma. Dose – 10 ml (250 mg) every 12 hr of tetracycline hydrochloride activity as tetracycline base buffered with sodium hexametaphosphate.

SUMMARY

Two aqueous tetracycline syrups have been described. Each 5 ml contains 125 mg of tetracycline hydrochloride activity as tetracycline base buffered with either 125 mg sodium hexametaphosphate/5 ml or 125 mg ammonium polyphosphate/5 ml along with suitable sweetening agents, preservatives, antioxidants, suspending agents, dispersing agents, flavors, and dyes.

Tetracycline serum concentrations have been presented with a large number of volunteers using single-dosage regimens of 5 and 10 ml of each syrup and multiple-dosage regimens of 5 ml every 6 hr and 10 ml every 12 hr of each syrup.

Both syrups are excellently absorbed from the gastrointestinal tract and give therapeutic serum concentrations of tetracycline.

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EXCRETION OF DYES AND PENICILLINS G AND V FROM THE BOVINE UDDER

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For many years the presence of penicillin in milk, which results from use in the treatment of bovine mastitis, has been a common cause of failure in cheese production, as it can inhibit the acid production of "starter" organisms [1]. More recently the hazards attending human consumption of milk contaminated with penicillin have been emphasized [2,3] since penicillin has been shown to be an allergenic or sensitizing agent even in minute quantities. Still more recently another public health hazard has been recognized; the bacterial flora of penicillin-contaminated milk may be changed so as to allow toxin-producing penicillin-resistant staphylococci to develop [4].

In order to prevent the entry of penicillin into the milk supply the following measures have been tried: (a) Prohibition of the use of milk for human consumption for a certain period after the last dose of penicillin [5]; (b) Labeling of veterinary preparations with warnings against the improper use of milk for a certain period after the last dose of penicillin; (c) Educational campaigns stressing the dangers of improper use of milk containing penicillin; (d) Penalizing action by processors against suppliers of milk found to be contaminated with penicillin [6]; (e) Dyeing of penicillin preparations for the treatment of mastitis, so that contaminated milk may be readily detected by its color, [7,8,9,10,11]; and (f) Limitation of the strength of penicillin in preparations for the treatment of mastitis [12].

The object of the present work was to find more suitable dyes and penicillins for use in intramammary infusions, to study the rate of excretion of low doses of dyed and undyed preparations, and to examine the effect of the vehicle on their rate of excretion from the milk.

MATERIALS AND METHODS

The dyes used in the animal experiments were fluorescein LTS (water-soluble), fluorescein S (oil-soluble), and brilliant blue FCF (disodium salt).

Micronized procaine penicillin G; and micronized penicillin V potassium were each suspended in a "water-repellent" mixture of peanut oil and aluminium stearate (R), a mixture of liquid paraffin and white soft paraffin (P), and a mixture of (P) and Tween 80 (T).

Healthy Red Poll cows at various stages of lactation were used in the experiments. Most of the experiments were in Latin square designs, the number of cows, replicates, and experimental periods being multiples of the number of preparations to be tested.

The preparations, dispensed in collapsible tubes in quantities of 0.75 to 3.0 g, were deposited in the teat canal after the evening milking and massaged into the cistern. In most of the experiments the same preparations were used in each quarter, the milk taken subsequently from the four quarters being pooled at each milking. The cows were milked twice daily by machine and the pooled milk was weighed.

The samples were stored over dry ice until assayed, this occurring within a few days. The penicillin content was determined by a cylinder-plate diffusion method using Sarcina lutea SKF 1360 in comparison with standards of penicillins V or G. The standards and, where necessary, the samples were diluted in normal milk demonstrated to be free from inhibitory substances. This method allows estimation of penicillin down to 0.01 units/ml.

The concentrations of dye were estimated by the number of dilutions required to reduce the color to below visual detection (2.3 ppm) in the case of the fluoresceins and by comparison with known concentrations in milk in the case of brilliant blue FCF.

RESULTS

Trial using fluorescein. Proceeding in a manner similar to that of earlier investigators [7,8], suspensions of procaine penicillin G in vehicle R were prepared which also contained fluorescein LTS, fluorescein S, or a mixture of both dyes in equal parts. The proportions of dye to penicillin were 150, 250, 350, or 450 mg/100,000 units. Each of the twelve preparations was injected into each of two quarters, six cows being used for the whole experiment. The milk from each quarter was examined for dyes and for penicillin at each of the subsequent ten milkings.

From the average percentage recovery of dyes and penicillins it can be seen that the correlation between dye and penicillin was fair in the case of fluorescein LTS but poor in the other two cases. Table 1 shows that fluorescein S and the mixture of fluorescein LTS and S tend to be excreted for a longer period into the milk than the penicillin. It was noticed that dye sometimes reappeared in a later milking after having been absent from an earlier one. The dyes were also detected in the urine, showing that absorption from the udder had occurred.

Table 1. Extinction End Points of Fluorescein and Procaine Penicillin G Dye Treatment*

Substance	Number of cows	Milking at which extinction occurred											
		Fa†	Fb	Fc	Fd	Ua	Ub	Uc	Ud	UFa	UFb	UFc	UFd
Penicillin	1	6	8	7	6	6	6	6	6	3	4	4	3
Dye	1	7	9	9	9	6	5	6	6	5	6	5	5
Penicillin	2	6	5	5	5	5	5	5	4	5	5	5	5
Dye	2	8	6	5	7	5	5	5	5	7	7	7	9

*Milking at which dye disappeared and at which penicillin dropped below 0.1 units/ml.

†F = fluorescein S. (oil-soluble); U = fluorescein LTS (water-soluble); UF = equal mixture of fluorescein S and fluorescein LTS; a = 150 mg dye/100,000 units procaine penicillin G; b = 250 mg dye/100,000 units procaine penicillin G; c = 350 mg dye/100,000 units procaine penicillin G; d = 450 mg dye/100,000 units procaine penicillin G.

Coloring of milk by various dyes. Because of the limitations of the fluoresceins and because of their possible toxicity a search for more suitable and harmless dyes was made. A number of the more stable food dyes such as erythrosine, brilliant black, acid violet, rhodamine B, and brilliant blue FCF were added to milk to find the lowest concentration at which they could be detected visually. Brilliant blue FCF was the most suitable, being detectable at 0.25 ppm without reference to a milk control and was, therefore, selected for further investigation.

Preliminary trials showed that while 100% of the dye was recovered only 50% of the penicillin was recovered. Since the limit of detection of dye was 0.25 ppm and the desired limit of detection of penicillin was 0.1 unit/ml, brilliant blue had to be added to preparations at the rate of 125 mg/100,000 units for a proportional recovery to be obtained.

Trials using brilliant blue FCF. Brilliant blue FCF was suspended together with procaine penicillin G in vehicles R and P, the proportion of dye to penicillin being 125 and 94 mg/100,000 units, respectively. Two cows recived 120,000 units of the R preparation, two received 30,000 units of R, and two received 30,000 units of P in each quarter. The average percentage recoveries in Table 2 show good correlation between dye and penicillin.

Table 2. Average Percentage Recoveries of Brilliant Blue FCF and Procaine Penicillin G

Penicillin, units/quarter	Base	Substance recovered	Milkings						Total
			1	2	3	4	5	6	
100,000	R	Penicillin	40.3	8.41	0.49	0.26	0.04	0.01	49.51
		Dye	86.3	20.4	4.6	0.53	0.4	0.16	112.39
25,000	R	Penicillin	56.1	2.19	1.11				59.40
		Dye	116.3	6.4	3.9	0.46			127.06
25,000	P	Penicillin	56.4	5.1	0.87	0.12			62.49
		Dye	130.0	14.8	2.7				147.50

A second trial was planned using procaine penicillin G suspended in vehicle R, with and without the addition of brilliant blue FCF at the rate of 125 mg/100,000 units. Five cows received 120,000 units and five received 30,000 units of the dyed preparation and ten cows received similar doses of the undyed preparation in each quarter. The correlation between dye and penicillin recoveries was poorer than was observed in the earlier trial.

As it was thought that these disappointing results might be due to the 20 cows giving poor yields of milk, further work planned on these cows was abandoned and 14 cows giving better yields were substituted. Of these, seven were given 120,000 units of the dyed preparation and the others were given the same dose of the undyed preparation in each quarter. Again, there was poor correlation between dye and penicillin, the dye tending to be excreted for a longer time than the penicillin. Closer examination of the preparations and the milk showed that some of the dye was present in micelles, probably in close association with aluminium stearate, and that it was these isolated but intensely stained micelles which were often responsible for imparting a color to samples of milk taken a long time after administration of the dose. These findings are in accordance with those of Rasmussen and Simesen [9].

In the light of these findings a further trial was planned to compare the excretion of brilliant blue FCF and penicillin in vehicles R, P, and T (containing 1.0% of Tween 80) as it was thought, following the work of Rasmussen and Simesen, that a surface-active agent may accelerate the rate of excretion of dyes and penicillin. Also, in this trial, it was decided to use micronized dye, so as to diminish the tendency for large particles to become embedded in micelles when vehicle R was used. In each case the proportion of dye to procaine penicillin G was intended to be 125 mg/100,000 units but analyses showed a small variation

Table 3. Effect of Base on Dye-Penicillin Relationship, Average Percentage Recovery of Brilliant Blue FCF and Procaine Penicillin G

Penicillin, units/quarter	Base	Substance recovered	Milkings							Total
			1	2	3	4	5	6	7	
100,000	R	Penicillin	27.0	4.71	1.47	0.52	0.13	0.03		33.86
		Dye	55.5	8.7	3.1	1.0	0.66	0.31		69.27
100,000	P	Penicillin	31.9	7.81	0.26	0.08	0.03			40.08
		Dye	50.6	11.5	0.95	0.29	0.13			63.47
100,000	T	Penicillin	43.4	6.08	0.50	0.07	0.01			50.06
		Dye	73.0	15.4	1.7	0.43	0.04	0.02		90.59
25,000	R	Penicillin	29.5	6.15	1.61	0.20				37.46
		Dye	46.4	11.2	4.8	1.8	1.0			65.20
25,000	T	Penicillin	36.8	5.61	0.71	0.17				43.29
		Dye	65.0	15.4	2.0					82.40

Table 4. Comparison of Rates of Excretion of Procaine Penicillin G and Penicillin V, Average Percentage Recovery

Penicillin	Average dose, units/quarter	Base	Milkings								Total
			1	2	3	4	5	6	7	8	
G	113,000	R*	39.5	11.6	4.00	0.93	0.30	0.11	0.05	0.03	56.56
G	27,700	R*	42.1	12.9	2.67	0.66	0.27	0.08	0.03	0.01	58.80
G	25,300	P*	48.5	8.85	1.73	0.54	0.18	0.04	0.02	0.01	59.88
V	25,600	R*	22.4	4.79	1.71	0.69	0.32	0.19	0.14	0.05	30.27
V	24,900	P*	32.4	5.48	1.21	0.37	0.26	0.11	0.10	0.10	40.00
V	28,200	P†	25.2	6.10	2.47	0.53	0.36	0.24	0.17	0.12	35.16

*in 1-ml tube.
†in 3-ml tube.

occurred in both P and T preparations. A trial using bases R, P, and T and 100,000 units/quarter was conducted in six cows for three weeks in the form of duplicated 3 × 3 Latin squares. Another trial using bases R and P and 25,000 units/quarter was conducted in six cows over two weeks in the form of duplicated 2 × 2 Latin squares.

The results in Table 3 show good correlation between dye and penicillin excretion for all preparations, R being better than P which was better than T. Excretion was distinctly slower with the R preparation and again the dye, although micronized, tended to persist in discrete micelles in the milk from cows given R.

Compatibility of dyes with penicillin. Procaine penicillin G suspended in vehicle R lost about a quarter of its potency during storage for 14 months at 30 C, whereas in the presence of fluorescein LTS or fluorescein S over half the potency was lost during storage at 5 C for the same period.

Procaine penicillin G suspended in vehicle R together with brilliant blue lost no potency during storage at 25 C for six months.

Trials using penicillin V potassium. An experiment was carried out to compare the rate of excretion of the freely soluble potassium salt of penicillin V with that of the sparingly soluble procaine salt of penicillin G suspended in different vehicles. The experiment was conducted in the form of a 6 × 6 Latin square, six cows being used on each of six preparations in each of six experimental periods. The preparations and doses are indicated in Table 4 together with the average recoveries of penicillin in the milk.

It is seen from Table 4 that the dose of penicillin had little effect on the proportional rate of excretion, that the rate of excretion from vehicle R was surprisingly little slower than that from vehicle P, and that the rate of excretion of penicillin V potassium, especially when suspended in a relatively large volume of vehicle, was surprisingly slower than that of procaine penicillin G. Also, the total percentage recovery was lower for penicillin V potassium.

The trial was partially repeated and extended to include a comparison of a dye preparation of penicillin V potassium. Procaine penicillin G was suspended in vehicle R and penicillin V potassium in vehicle P; in the latter case with and without the addition of micronized brilliant blue FCF in the proportion of 31 mg/100,000 units. The trial was conducted in the form of triplicated 3 × 3 Latin squares, nine cows being used in each of the 3 preparations in each of 3 experimental periods. The dose of procaine penicillin G was 96,000 units in each quarter and that of potassium penicillin V was 23,000 units in each quarter.

Table 5. Comparison of Rates of Excretion of Procaine Penicillin G and Penicillin V Potassium With and Without Dye, Average Percentage Recovery

Penicillin	Base	Average dose units/quarter	Milkings						Total
			1	2	3	4	5	6	
G	R*	95,600	39.3	9.81	3.10	1.00	0.21	0.05	53.47
V	P*	22,100	20.1	6.56	2.56	1.74	1.07	0.43	33.43
V	P* plus dye†	23,000	24.3	5.74	2.52	1.45	0.61	0.30	34.93

*in 3-ml tube
†Brilliant blue FCF, 31 mg/100.000 units.

The results in Table 5 show that the dye has little effect on the rate of excretion of penicillin V potassium and confirm that this salt is excreted more slowly than procaine penicillin G.

Antibacterial activity of penicillin V potassium. A strain of Streptococcus agalactiae and one of Staphylococcus mastitis, both of which were inhibited by 0.10 unit/ml of procaine penicillin G, were also inhibited by 0.08 unit/ml of penicillin V potassium when tested on nutrient agar containing a closely spaced, graded series of concentrations of these penicillins.

Solubility of penicillin V potassium in milk. Since milk contains calcium ion, and since penicillin V calcium is sparingly soluble, it was of interest to determine whether penicillin V potassium would dissolve in milk and remain in solution or whether it would be precipitated. It was found that penicillin V potassium could be dissolved to the extent of 2.0% w/v or more without precipitation occurring.

DISCUSSION

Interpretation of the data summarized in Tables 2 and 3 will be assisted if it is borne in mind that for cows giving normal milk yields of, say, 1 liter per quarter per milking and receiving 100,000 units of penicillin and 125 mg of brilliant blue FCF per quarter, a recovery of 0.2% of the dye and 0.1% of the penicillin in a given milking represents a concentration in the milk of 0.25 ppm of dye and 0.1 units/ml of penicillin. The concentrations represent the limit below which the dye cannot be detected visually and the limit below which penicillin is of comparatively little practical importance since milk contaminated with penicillin is usually diluted with considerable quantities of normal milk before reaching the cheese factory or the consumer.

The data show that brilliant blue FCF is a suitable dye for coloring mastitis preparations which do not contain water-repellent substances such as aluminium stearate. With vehicles free from such substances the rate of excretion of the dye was well correlated with that of penicillin and the milk was also colored blue only for as long as it contained appreciable quantities of penicillin. Brilliant blue FCF has the advantage over dyes proposed by earlier workers since it is a certified food color and accidental ingestion of milk containing traces of color would not be dangerous and also since it can be detected visually in ordinary light at a very low concentration. Use of brilliant blue FCF in mastitis treatments in the proportion of 125 mg/100,000 units of penicillin is advocated as a practical way of warning farmers, inspectors, and milk processors that a batch of milk may be seriously contaminated with penicillin.

The over-all incidence of penicillin in milk could be reduced by reducing the normal dosage rate for the treatment of mastitis. In has been clearly shown by Murnane [13] and many other workers that three doses of 25,000 units of penicillin in oily or aqueous vehicles given at daily intervals is an effective treatment for streptococcal mastitis and that even very much higher and more frequent doses are relatively ineffective for the treatment of staphylococcal mastitis. There seems no justification, therefore, for the widespread use of doses as high as 500,000 units. Indeed, apart from the convenience of being able to administer only one or two doses instead of three there seems to be little justification for the very well-established practice of giving doses of 100,000 units of procaine penicillin G in a water-repellent vehicle.

The unexpected finding that penicillin V potassium is excreted more slowly than procaine penicillin G is of interest from two angles. First, it suggests that a dosage regimen comprising two doses of 25,000 units 48 hr apart may be a suitable treatment for streptococcal mastitis, giving the farmer some of the convenience of 100,000 units doses of procaine penicillin G and at the same time reducing the potential contamination of the milk supply by reducing the total quantity of penicillin used in the dairy industry. Secondly, when it is considered that the total recovery of penicillin V potassium in the milk is less than that for procaine penicillin V is partially absorbed through the udder into the bloodstream and hence may well be brought into more intimate contact with infected tissues than is the case for penicillin G. If this were so, then frequent administration of high doses of penicillin V might be an effective treatment even for staphylococcal mastitis.

ACKNOWLEDGMENTS

Our thanks are due to Imperial Chemical Industries of Australia and New Zealand Ltd. and the State Laboratories of Victoria for supply of the dyes. The trade name of brilliant blue FCF is Edicol Supra Blue EGS.

We are also grateful to Mr. O. St. J. Kent, Principal of the School of Dairy Technology and to Mr. V. F. Davey, Consultant in Antibiotics at the Commonwealth Serum Laboratories for helpful advice and encouragement during the investigation.

SUMMARY

In order to reduce contaimination of milk by penicillin three methods of approach to the problem have been investigated.

First, a search was made for a harmless dye, the excretion rate of which is closely correlated with that of penicillin. Brilliant blue FCF was found to be more suitable than those studied by earlier investigators. This dye, used at the rate of 125 mg/100,000 units of penicillin in mastitis preparations will permit easy detection of penicillin-contaminated milk. Dye can be successfully added to both 100,000- and 25,000-unit preparations if the vehicle does not contain water-repellent substances.

Second, believing that over-all contamination of the milk supply may be reduced substantially by use of much lower doses of penicillin than those now common, the rate of excretion of low doses of dyed and undyed preparations was studied.

Third, comparisons were made of excretion rates of soluble and insoluble salts of penicillin V and G dispensed in bases containing, or free from, dyes and water repellent substances.

It was found that low doses of the soluble potassium salt of penicillin V, dispensed in a paraffin base with or without brilliant blue FCF were excreted surprisingly slowly. Factors affecting the therapeutic efficacy of such preparations are considered.

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CLINICAL RESULTS WITH SPIRAMYCIN*

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Some of the greatest advances in modern medicine have been made in the treatment of infections and these have been due to the discovery and development of antibiotics.

Penicillin was the first antibiotic to be produced in quantity and used widely. It was followed by newer antibiotics which were used in infections where penicillin was not effective or where strains resistant to penicillin had developed. The occurrence of new resistant strains was recognized during the treatment of some parasitic infections in the past.

New antibiotics were needed, discovered, produced, and used. Streptomycin and other broad-spectrum antibiotics have been used extensively and one after another have produced resistant strains, thus necessitating research for new bactericidal agents.

The present state of affairs is shown by the increasing number of reports of recurrent furunculosis, carbuncles, secondary respiratory tract infections, secondary wound infections in hospitals, and fatal cases of septicemia. The mechanism and history of development of resistant strains of staphylococci are well covered in these reports. Hospital surveys reveal that many apparently healthy carriers are the reservoir for these strains. The nasopharynx of the carriers and patient-infected mattresses, walls, and floors are the sources of these infections. Staphylococci isolated from these sources were resistant to penicillin in 90% of the cases, resistant to streptomycin in 30%, and resistant to the broad-spectrum antibiotics in about 20% of the cases.

In view of this we have tried a new antibiotic, spiramycin, in various cases of staphylococcal infections presenting different clinical manifestations.

Spiramycin is the antibiotic obtained from Streptomyces ambofaciens. It is a mixture of organic bases with the approximate empirical formula, $C_{22-24}H_{34-44}O_{7-8}N$.

The Handbook of Biological Data [1] gives the following information about spiramycin: "Principally active against Gram-pos. bacteria, particularly staphylococci, resistant to the usual antibiotics. Inhibits streptococci, corynebacteria, Neisseria and certain strains of clostridia. Also active against some rickettsiae. Little activity against myco- and enteric bacteria. Clinical: Effective against respiratory, staphylococcal, streptococcal and gonococcal infections, some virus pneumonia and exanthematic typhus."

The studies done on spiramycin show a peculiar discrepancy between the experiments in vitro and in vivo. Spiramycin required higher concentrations than other antibiotics of the same group, such as erythromycin and carbomycin, to inhibit the growth of bacteria in vitro, whereas the opposite was true in vivo. This is true in animal experiments as well as in humans. The blood levels are of the same order as those of other antibiotics. Why is it then, that spiramycin is more effective in vivo than the others?

Previous workers [2, 3, 4, 5, 6] have shown that infected mice needed less spiramycin than erythromycin, carbomycin, oleandomycin, or triacetyloleandomycin and the animals treated with spiramycin have survived, on the average, those treated with the other antibiotics. While studying the distribution of spiramycin in the body they noted it was retained in high concentration in the tissues. They reported that 6 hr after oral administration, Spiramycin has the highest concentrations in most of the organs; its level is 20 times higher than that of erythromycin in the myocardium and 50 times that of carbomycin in the liver. In the kidneys the concentration of spiramycin is twice that of erythromycin or oleandomycin and seven times that of carbomycin. They also noted that the difference becomes very important 24 hr after administration. Spiramycin maintains its 6-hr levels in all the viscera and it is almost impossible to detect any trace of the other antibiotics in the organs. Spiramycin is still detectable in the organs 48 hr after a single oral administration and the levels reached are higher than the highest attainable spiramycinemias. The administration of a single oral dose of spiramycin protected the mice against an intravenous fatal dose of staphylococci given at the time when the blood level of spiramycin was already at a low ebb. That is the reason why several authors state, as Spitzy and Hitzenberger [7] do, that the tissue concentration is more important than that of the plasma.

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*Trade Mark: Rovamycine, Poulenc Ltd., Montreal.

We have more than three years experience with spiramycin. We have been administering 2 to 4 g of spiramycin in divided daily doses to 86 cases and have had excellent results with respiratory infections where the main pathogenic agents were staphylococci and streptococci. Many cases of staphylococcal infections of the skin and hydradenitis responded to the same treatment with complete healing and no failure. We had only three cases of "flared up" chronic tonsilitis where there was no response and we had to resort to tonsillectomy.

Some time ago we started to treat our patients with a daily 2-g dose of spiramycin given in the morning. The results are illustrated in Table 1.

Table 1. Treatment of Various Diseases With 2-G Doses of Spiramycin Given Daily for Three Days

Disease	Cases	Results		
		Good	Fair	None
Staphylococcal infections of the skin (hydradenitis, furuncles, carbuncles, lymphadenitis, sycosis barbae)	23	19	3	1
Upper respiratory infections	12	12		
Otitis	2	2		
Tonsillitis	8	8		
Bronchopneumonia	6	5		1
Total:	51	46	3	2

When we compared the results obtained with the divided doses with those obtained with a single dose we saw that the responses were very similar. The advantage of the single daily dose is that a smaller quantity is needed and the patient has to take the drug only once a day. Both dosage methods give a high tissular concentration, which explains the good clinical results.

SUMMARY

Spiramycin, a relatively new antibiotic, has already been used in the various laboratories of research centers and hospitals as well as in clinical practice.

Its principal use is against staphylococci and streptococci and a series of various pathogenic agents such as Hemophilus pertussis and Rickettsia.

Earlier reports have indicated that the antibiotic was present in serum at concentrations imcompatible with the excellent results obtained in daily practice. Such results can now be explained by the high and long-lasting tissue concentrations which follow absorption of spiramycin.

On the basis of recent laboratory studies, which showed that spiramycin had a peak absorption in tissues after 24 hr and was still present in appreciable concentrations after 48 hr, we gave 51 patients 2 g of spiramycin in a single daily dose and compared the clinical results with those obtained following divided doses. The single daily dose produces the same therapeutic results as the divided dosage.

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COMBINED ANTIBIOTIC AND ANTIFUNGAL THERAPY

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INTRODUCTION

Coincident with the increased use of antibiotics, fungal infections have been encountered with greater frequency. It is conservatively estimated that mycoses are the direct cause of some 350 to 450 deaths each year [1] in the United States, a mortality greater than that of whooping cough, diphtheria, scarlet fever, typhoid fever, and several other clearly defined diseases. While morbidity figures for the mycoses are not available, it is axiomatic that they are frequently encountered in all types of medical practice and are a complicating factor in many illnesses. Yet the physician has at his disposal only a few fungicidal agents, in comparison to the many antibacterial drugs available.

For the treatment of mycotic infections it is desirable that the agent be both fungicidal and bactericidal since fungus infections are frequently complicated by bacterial invasion. Similarly, treatment of bacterial infections may be thwarted by an accompanying fungus. The need for an agent effective against both infections, with the accompanying qualitities of ease of administration and absence of irritation, clearly exists.

The fungicidal properties of a number of fatty acids are well established and it was felt that fatty acid derivatives of a stable antibiotic might possibly provide the desired combination of bactericidal and fungicidal actions. Therefore, a systematic survey of the fatty acid derivatives of neomycin was undertaken. As is the case with the acids alone [2], the derivatives incorporating long-chain acids proved to be more active than those of lower molecular weight, with neomycin undecylenate exhibiting especially interesting properties.

EXPERIMENTAL

Neomycin undecylenate is a waxy unctious powder with a definite candle-like odor. It is stable and blends with lipids easily. It has great substantivity and will cling to the skin or to hair and cloth fibers for long periods of time. An aerosol containing 0.2% neomycin base was used for application on the skin.

Infected bruises and lacerations. The compound was first used in wounds complicated by mixed infections which would give it the best opportunity to demonstrate its versatility. In 164 cases, the majority of which had resisted soaks with magnesium sulfate and Burow's Solution, the aerosol spray was applied several times daily with the following responses: excellent, 57; good, 105; fair, 1; poor, 1. Control of infections was rapid in all patients with the exception of the one whose response was classified as poor. Even this case, however, appeared to respond better to neomycin undecylenate than to previously administered Burow's Solution.

Burns. In 302 cases on burns, all of the second or third degree and including ten cases involving more than 50% of the body area, the aerosol spray containing neomycin undecylenate was applied. In certain patients having two or more distinct, burned areas of sufficiently small size to make the method practical, control was established by treating some areas with neomycin undecylenate and others with petrolatum and pressure, antibiotic ointments, and other standard methods. The areas left open and treated with the aerosol spray responded most rapidly and satisfactorily. Results in 302 patients were as follows: excellent, 26; good, 267; fair, 7; poor, 2.

It should be noted that five of the above patients, having burned areas covering from 60% to 85% of the body surface, expired. The value of the medicament is based, however, upon its ability to control skin complications and was considered good in three of these death cases, fair in one, and poor in one. Fluid therapy and other appropriate measures were utilized as required. Aerosol spray was applied once each 4 hr and allowed continuous observation of the burn sites. The menstruum permits continuing contact of the medication to the infected areas and avoids collection of serum under the medication; it seems to breathe and allow vapor to pass through.

Skin graft preparation. In situations where skin grafts were required (21 cases) and it was desirable to make such grafts without undue delay, the aerosol spray was used to clear mixed infection from the graft site with the following results: excellent, 4; good, 15; fair, 1; poor, 1.

In vitro studies. The initial clinical impression being highly favorable, it was decided to do in vitro studies as a measure of the compound's effect against specific organisms. Because of the insolubility of the compound these studies proved quite difficult and many agents were tried to no avail. Finally, a coordination compound was formed which provided an excellent solution and it later developed that the agent potentiated the action of neomycin undecylenate. Later, clinical tests of the potentiated compound showed that the increase in both bactericidal and fungicidal properties was marked.

In vitro studies were performed by titration in the media customarily used to grow the organism concerned. The cultures were those of recent human source except for a few uncommon organisms which are obtained from other laboratories. Results are expressed in μg/ml of potentiated neomycin undecylenate necessary to inhibit growth. It should be remembered that the figures are given in total weight of potentiated compound and that, roughly, only 14% of this weight is neomycin base. Each test represents ten or more determinations, each being checked from two sources. Table 1 indicates the results with bacteria and Table 2 the results with fungi; comparative results obtained with neomycin palmitate and neomycin sulfate are shown.

Simultaneous with the in vitro testing, the coordination compound undergoing laboratory evaluation was tested in patients. It was noted, as previously mentioned, that a significant potentiating effect has been achieved. Being more water soluble than the untreated compound, the neomycin undecylenate comes out of compound in the form of a waxy film on the surface being treated. Because the agent is effective in rather small concentrations, numerous applications result in prolonged antimicrobial action. Development of microbial resistance to the potentiated compound is infrequent, as with neomycin, and the spectrum of the potentiated compound is broader than that of neomycin toward bacteria and of undecylenic acid toward fungi. Considering the small fraction of the total compound represented by the totals of neomycin and undecylenic acid, it may be said that the antibacterial and antifungal actions of the two substances have been potentiated tenfold.

The potentiated material was prepared in three dosage forms; dusting powder containing 0.35% neomycin base, tablets containing 500 mg of neomycin base,

Table 1. Comparative Minimal Inhibitory Concentrations, μg/ml, of Neomycin Undecylenate, Palmitate, and Sulfate Necessary to Inhibit Growth

Bacteria	Undecylenate	Palmitate	Sulfate
A. aerogenes	1.56	1.56	3.12
C. diphtheriae	0.10	0.10	0.10
S. typhosa		3.12	12.5
E. coli	1.56	6.25	12.5
H. influenzae	6.25	25.0	6.25
H. pertussis	1.56	6.25	1.56
K. pneumoniae	1.56	1.56	3.12
P. vulgaris	1.56	25.0	6.25
P. aeruginosa	3.12	12.5	12.5
S. schottmuelleri	6.25	6.25	25.0
S. paradysenteriae	1.56	25.0	12.5
M. pyogenes albus	0.78	0.78	0.78
M. pyogenes aureus	1.56	1.56	3.12
M. pyogenes citreus	1.56	3.12	6.25
M. pyogenes epidermidis	1.56	1.56	0.39
S. viridans	0.78	3.12	100
S. hemolyticus	0.78	0.78	6.25
S. pyogenes	0.20	0.20	1.56
S. faecalis	3.12	50	100
D. pneumoniae	12.5	1.56	12.5
C. perfringens	3.12	3.12	250
S. aureus			
coagulase-negative	0.78		
coagulase-positive	1.56		
S. albus	0.78		
Enterococcus	0.78		
E. intermedium	50		
Alpha streptococcus	0.78		
Para-coli intermedium	25		

and vaginal suppositories containing 0.36% neomycin base. With these additions to the armamentarium, a variety of conditions were treated and topical, vaginal, and oral routes of administration were employed.

Topical applications. These included decubitus and varicose ulcers, miliaria, severe dermatitis, fistula, and dermatophytosis. Aerosol application was used in all cases, with dusting powder also employed where indicated. All these patients had been previously treated by standard therapeutic measures or served as their own controls by having certain areas of skin treated with antibiotic creams, dusting powders, or steroid foams simultaneous with neomycin undecylenate treatment. Results showing neomycin producing improvement equal to or better than standard methods are indicated in Table 3.

All patients with dermatophytosis of the foot had been treated previously with permanganate soaks or caprylic or propionic preparations. Twenty-four of them had been treated with fungistatic antibiotics with poor results. Cures in this condition are considered no better than imaginary because of the constant reinfection from shoes and environment and relief from cracking, itching, and other symptoms was the criterion of success. After initial treatment with aerosol

Table 2. Minimal Inhibitory Concentrations, μg/ml, of Potentiated Neomycin Undecylenate Necessary to Inhibit Growth

Fungus	Undecylenate	Palmitate	Sulfate
Achorion schoenlini	5	100	500
Candida albicans	200	500	500
Cryptococcus neoformans	15.6	500	500
Microsporum audouini	5	15.6	125
Microsporum gypseum	5	15.6	500
Sporotrichum schenckii	25	62.5	500
Trichophyton mentagrophytes	50	31.2	250
Trichophyton rubrum	31.2	62.5	500
Trichophyton tonsurans	62.5	500	500
Hormodendrum pedrosoi	5	15.6	500
Allescheria boydii	15	62.5	500
Microsporum canis	5	31.2	500
Torulopsis utilis	31.5	62.5	500
Rhizopus nigricans	5	62.5	500
Aspergillus fumigatus	5	62.5	500
Blastomyces dermatotidis	62.5	200	500
Torula histolyticus	62.5	200	500

Table 3. Topical Application of Neomycin Undecylenate

Indication	Dosage form	Excellent	Good	Fair	Poor	Total
Decubitus ulcer	Aerosol, dusting powder	3	16	0	0	19
Varicose ulcer	Aerosol, dusting powder	5	35	1	0	41
Miliaria	Aerosol, dusting powder	9	24	9	8	50
Severe dermatitis	Aerosol, dusting powder	4	36	3	2	45
Fistula	Aerosol	5	19	0	0	24
Dermatophytosis (foot)	Aerosol, dusting powder	0	140	21	7	168
Dermatophytosis (groin)	Aerosol, dusting powder	2	7	1	0	10

spray, patients were directed to use the dusting powder in their shoes once each week. One hundred and sixty patients who followed this regimen were followed for six months and no recurrence of the active infection was found.

Twenty cases were selected with advanced dermaphytosis of both feet in which the organism was identified in scrapings. In ten cases of this series, one foot was treated with neomycin undecylenate powder and the other with powder containing 25% zinc undecylenate. The same conditions as to bathing, stockings, and shoes were maintained. The powder was applied morning and night and the treatment applied for 10 days. On the side that was treated with neomycin undecylenate, symptoms were relieved in 24 hr and scraping were negative in 5 days. On the side treated with the zinc undecylenate, symptoms were relieved in 48 hr and scrapings were negative in 8 days. In a six-month follow-up none of the sides receiving the neomycin undecylenate showed recurrence, while recurrence requiring additional therapy was evident on four of the sides receiving the zinc undecylenate.

In another series of ten cases one side was treated with neomycin undecylenate and the other foot was treated with a powder containing 33.33% triacetin. On the side treated with neomycin undecylenate all symptoms were relieved in

24 hr and the scrapings were negative in 5 days. The feet treated with triacetin did not show relief until the third day and scrapings were negative on the ninth day. In six months there were no recurrences on the neomycin undecylenate-treated feet. On the sides treated with triacetin there were three recurrences caused by the organisms demonstrated on scraping.

Vaginal administration. As with dermatophytosis of the foot, vaginal infections can rarely be considered cured as the result of any specific treatment. Fungal and protozoan infections may be neutralized or even eliminated but proximity to the rectum provides a constant source of reinfection, making constant prophylaxis necessary. Neomycin undecylenate has a distinct lack of irritation in long-term use and does not appear to allow the emergence of resistant strains, making it well suited for such prophylactic use. Cultures were done on the patients in this series and they were placed on a regimen of one suppository each week. Recurrence was found in only five of 97 cases as shown in Table 4.

Table 4. Response to Vaginal Administration of Neomycin Undecylenate

Primary organism	Excellent	Good	Fair	Poor
Trichomonas vaginalis	0	60	1	2
Bacteria	0	23	1	2
Fungus	0	7	0	1

Oral administration. Forty-one patients were prepared for colon surgery by administration of 500-mg tablets of neomycin undecylenate four times daily for 3 days. Bacterial cultures revealed that the intestinal sterilization achieved was as good as had been contained earlier in a series of several hundred patients given neomycin sulphate plus sulfathelidine. These tablets were also used in six patients with thrush with uniformly good results in all.

Side effects and sensitivity. Throughout the clinical trials checks for side effects were made both by observation and detailed questioning of patients. None were found which is not surprising considering the small amount of neomycin employed. Sensitivity and delayed-sensitivity patch tests were performed in 25 patients to measure development of irritation. Grade 1 reactions were obtained in two of these 25 patients while the remainder were negative. It may be concluded that in the dosages employed in this study the compound is virtually free of side effects.

SUMMARY

Clinical experience with neomycin undecylenate is reported. The compound shows combined bactericidal and fungicidal activity. A potentiated form of the compound has demonstrated both bactericidal and fungicidal effect in vitro and has been used with good effect in topical applications, for intestinal antisepsis, and as a vaginal prophylactic. In mixed infections neomycin undecylenate is effective in smaller doses than is neomycin sulfate, serving to reduce whatever dangers of toxicity exist with use of the sulfate.

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FATTY ACID PRODUCTION BY FLORA OF THE AXILLAE AND INHIBITION BY DEODORANTS

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INTRODUCTION

The reports in the literature of bacteria responsible for axillary odors [1-5] prompted us to study the flora of the axillary region, fatty acids produced by them, and the effect of deodorants on these microorganisms.

The role of bacteria in axillary odor was demonstrated [6] when no odor change developed in perspiration filtered through a Berkefeld filter. Odor values increased, however, when unfiltered perspiration was allowed to stand and filtered samples gave rise to malodors on subsequent inoculation with micrococci.

Because the axillae contain apocrine sweat glands which produce perspiration rich in organic material [7], microbial growth in these areas is expected. A study of the axilla of 29 Negro subjects [5] showed that a mixed flora was present and that filtered apocrine sweat, when inoculated with certain microorganisms and incubated at 37 C, developed an odor. Shelly and Cahn (1958) reported that micrococci (staphylococci) are the predominant organisms responsible for this odor. Kuno [8] reported a rather pleasant odor in which a pure culture of Aerobacter aerogenes was implicated. He attributed differences in odor rise to the differences in the resident bacterial flora. Fredell and Read [9] performed plate counts from the axillae although no attempts were made at identification of the bacteria isolated.

It was concluded [4] that the deodorant action is twofold in mechanism; there is an antibacterial effect thus preventing odor development and chemical change and the neutralization of odor itself occurs. Blank [10] showed that the aluminum salts used in deodorants were effective in vitro.

MATERIALS AND METHODS

Both the right and left axillae of 11 human subjects, male and female, White and Negro, were examined to determine the flora of the axillary region. Subjects were requested not to use an antiperspirant or deodorant and to refrain from bathing for at least 24 hr prior to our examination for microorganisms in the axillae.

Isolation of microorganisms. Sterile gauze pads (2 × 2) were moistened with approximately 4 ml of sterile saline and the axillae were swabbed using aseptic techniques. Both the right and left armpits were tested. The gauze pads were dropped into 250-ml Erlenmeyer flasks containing 100 ml of sterile trypticase soy broth* as well as flasks with a similar quantity of thioglycollate broth†. The broths were further subcultured into petri plates containing trypticase soy agar* to prevent antagonism of microorganisms and to avoid missing any cultures. After incubation at room temperature and 37 C, cultures were identified accor-

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*Baltimore Biological Laboratories, Baltimore, Maryland.
†Difco Laboratories, Detroit, Michigan.

ding to the Manual of Microbiological Methods [11] and named according to Bergey [12].

Subcultures were also made on Sabourauds dextrose agar for fungus determinations and the plates were incubated at room temperature (72-75 F) for 10 days.

Chromatography of fatty acids. Isolated cultures were grown in trypticase soy broth and in this medium containing 0.1% glycerol tributyrate. After 48 hr incubation at 37 C, the fermentation media were acidified with N HCl to pH 1, extracted with ethyl ether, alkalized with N NaOH, shaken, and the alkalized layer acidified. Repeated ether extractions were conducted. The ether was evaporated under reduced pressure* and the fractions taken up with absolute alcohol.

The extracts were added to Whatman No. 3 filter paper and fatty acids identified from prior chromatograms. Descending chromatography was employed using n-butanol—ammonium hydroxide mixtures (1 : 1) and bromocresol green or bromophenol blue as the indicator. R_f values [13] were established for the following fatty acids: formic, acetic, propionic, butyric, valeric, caproic, caprylic, capric, and lauric.

Deodorant testing. Bacterial cultures were maintained in broth [14] and after daily transfers, a 2-ml loop of an 18-hr culture was added to a test tube containing 20 ml of melted trypticase soy agar. The tubes were swirled and the contents were added to petri plates. Upon hardening, filter paper discs†, 2.4 mm in diameter containing approximately 50 mg of deodorant cream or 0.1 ml of liquid, were placed on the surface of the solid medium. Plates were incubated at 37 C for 24 hr and results were read in from the periphery of the disc to the edge of the zone of inhibition.

RESULTS

A mixed flora was shown to be present in 11 human subjects. Both the right and left axillae of subjects were tested. Individuals were requested not to use an antiperspirant or deodorant for 24 hr prior to examination in order to attempt

Table 1. A Comparison of Bacteria Isolated From the Left and Right Axillary Regions

Subject No.	Microorganisms recovered	
	Right axilla	Left axilla
1	*S. albus, S. aureus*	*S. albus, S. aureus, E. coli*
2	*S. albus, S. aureus*, Diphtheroids	*S. albus, S. aureus*, Diphtheroids
3	*S. albus, S. aureus*	*S. albus, S. aureus*
4	*S. albus, S. aureus*	*S. albus, S. aureus*
5	*S. albus, S. aureus*	*S. albus, S. aureus*
6	*S. albus, S. aureus, E. coli*	*S. albus, S. aureus, S. pyogenes, E. coli*
7	*S. albus, S. aureus, P. vulgaris, E. coli*	*S. albus, S. aureus, P. vulgaris, E. coli*
8	*S. albus, S. aureus, E. coli*	*S. albus, S. aureus*
9	*S. albus, S. aureus, S. pyogenes, E. coli, P. vulgaris*	*S. albus, S. aureus, S. pyogenes, E. coli, P. vulgaris, A. aerogenes*
10	*S. albus, S. aureus*	*S. albus, S. aureus, A. aerogenes*
11	*S. albus, S. aureus, A. aerogenes*	*S. albus, S. aureus, A. aerogenes*

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*Rotovap — Rinco Instrument Company, Greenville, Illinois.

†Schleicher & Schuell Co., Keene, New Hampshire.

Table 2. Subject Characteristics

Subject No.	Sex	Race	Deodorant user	Handedness	Type perspiration
1	M	W	No.	R	Moderate
2	M	W	Yes	R	Profuse
3	M	W	Yes	R and L	Moderate
4	M	W	No	R	Moderate
5	F	W	Yes	R	Moderate
6	F	W	Yes	R	Moderate
7	F	N	Yes	R and L	Moderate
8	F	W	Yes	R	Moderate
9	M	W	No	R	Sparse
10	M	N	Yes	R	Moderate
11	M	N	Yes	R	Moderate

to avoid inhibition of microorganisms. As can be seen in Table 1, a mixed flora was found to be present in the persons studied. All subjects (Table 2) were found to harbor both *Micrococcus* (*Staphylococcus*) *aureus*, and *Micrococcus albus*. *Escherichia coli* was found to be the next most common bacterium present, followed closely by *Aerobacter aerogenes*. Hemolytic streptococci of the *Streptococcus pyogenes* type were found to be present as were also diphtheroids. Hemolysis was determined on blood agar plates. *Proteus vulgaris* was shown to be present in two of the 11 subjects studied. It is also interesting to note that the bacterial flora in both the right and left axillae were essentially similar. Only in subjects numbered 6, 8, and 10 was an additional microorganism shown to be present in one of the axillary regions.

The *S. aureus* cultures isolated from various individuals were examined for coagulase activity. Only two of these were found to be coagulase-positive.

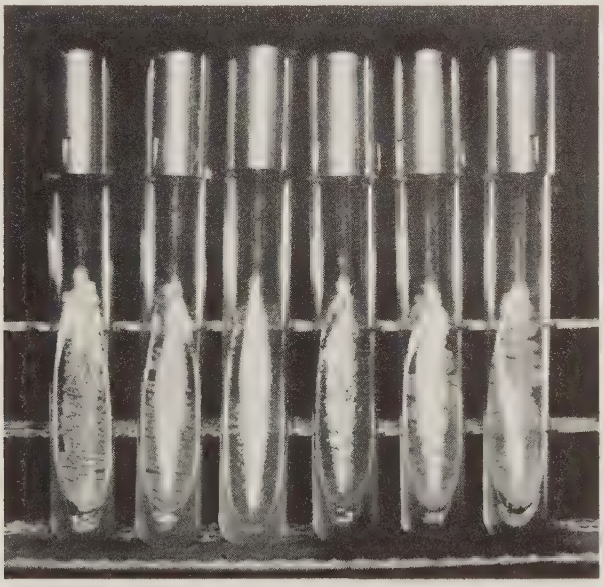

Fig. 1. Microorganisms isolated from the axillae of human subjects. From left to right the tubes contain *M. pyogenes* var. *albus*, *M. pyogenes* var. *aureus*, *S. pyogenes*, *P. vulgaris*, *E. coli*, and *A. aerogenes*.

Paper cromatrography seemed to indicate that butyric acid was produced by all of the aerobic microorganisms. The odor of butyric acid was especially perceptible in the fermented trypticase soy-tributyrin broths. Other fatty acids which appeared to be present from various bacteria were formic, acetic, propionic, and valeric.

Table 3. Effect of Various Deodorants on Bacteria Isolated From the Axillary Region

Deodorant type	Active ingredient	Zone of inhibition, mm					
		S. albus	*S. aureus*	*S. pyogenes*	*P. vulgaris*	*E. coli*	*A. aerogenes*
Liquid	Alcohol; Al chlorhydroxide	6	5	2	1	1	1
Cream	Al formate; Al chloride	4 and 2*	1 and 2*	3*	1 and 3*	1 and 3*	1 and 3*
Cream	Al sulfate	6*	5*	3*	4*	1 and 4*	3*
Cream	Al sulfamate	6*	4*	3*	2*	1*	1*
Liquid	Al chlorhydrate, methyl benzethonium chloride, G-11, and alcohol	10	6	2	1	2	1
Cream	Tyrothricin	0	0	0	0	0	0
Cream	Hexachlorophene	10	8	5	5	3	3

*Precipitate formed in medium.

DISCUSSION

The axillae contain apocrine sweat glands that are rich in organic matter. This material makes an excellent medium for the growth of microorganisms. Temperature and pH also favor the growth of bacteria in this area, hence a varied flora can be expected. One might raise the question then, from whence are these microorganisms derived Numerous workers [15, 16, 17] have shown that the skin harbors various microorganisms, thus they could readily pass from the cutaneous surfaces into the axillary regions. Bacteria that are normal to the enteric regions may be passed by the hands to the axillae. The microorganisms find conditions favorable and establish themselves as part of the normal flora of the axillary regions. The Staphylococcus as well as other microorganisms then attack the sweat, producing fatty acids or other compounds which may give rise to the off-odors of perspiration. Although a total of 66 microorganisms were isolated from the right and left axillae, only seven different species of microorganisms were identified. All of the aerobic microorganisms were able to produce fatty acids. These compounds are known to be malodorous. It would appear then that the result of this bacterial action would very well be responsible for axillary odors.

ACKNOWLEDGMENT

We wish to thank Mrs. M. G. Thompson and Mr. Philip Leidy for their extensive work in reviewing the literature and Dr. Cotty and his staff for chemical analyses.

SUMMARY AND CONCLUSIONS

A variety of bacteria were found to be present in the axillae of human subjects. Micrococci including *Staphylococcus albus* and *Staphylococcus aureus* were shown to be the predominant microorganisms present. Other microorganisms isolated included *Escherichial coli*, *Aerobacter aerogenes*, *Proteus vulga-*

ris, Streptococcus pyogenes and Diphtheroids. Microorganisms present in the right axilla were, in general, similar to those isolated from the left axilla of the same subject.

Butyric and other fatty acids were shown to be produced from flora of the axillae by paper chromatography.

Of various deodorant preparations tested, a formulation containing hexachlorophene showed the widest inhibition in trypticase soy agar.

No fungi were isolated from axillae.

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ORAL PENICILLIN (POTASSIUM PHENETHICILLIN)* IN GENERAL INFECTIONS

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INTRODUCTION

Since its discovery, penicillin has been accepted as the antibiotic of choice in most infections. The parenteral drug had disadvantages [1] and was followed by the development of various penicillin products for oral use. These preparations, however, have not been satisfactory to most physicians because of relatively low or unreliable blood levels. Even with the addition of a buffering agent, penicillin G concentrations in the blood are low and unpredictable. Penicillin V has the advantage of increased acid resistance over penicillin G, but the absorption of the former is only about 18% as judged by urine excretion studies [2]. An improved oral penicillin form, offering predictably higher blood levels, has therefore been required. Such a form would provide blood levels favorably comparable to those attained with the intramuscular forms.

The clinician is interested in obtaining maximal penicillin blood and tissue levels for their greater therapeutic effectiveness [3]. Maximal penicillin concentration in the blood stream provides greater possibility of exceeding relatively high minimum inhibitory concentrations (MIC) values required for bactericidal activity against certain organisms, and in most instances, provides a more prolonged contact of the antibiotic with the organism at effective levels.

A partially synthetic form of oral penicillin recently made commercially available is potassium phenethicillin. The drug has been shown to be acid stable and extremely soluble, affording rapid absorption from the gastrointestinal tract. High serum levels promptly effected by the oral administration of phenethicillin surpass those attained with other oral forms of penicillin, including phenoxymethylpenicillin potassium (penicillin V potassium) and those attained with intramuscular procaine penicillin G [4]. The new product consists of a mixture of two diastereoisomers, about 67% of which is the L-isomer. Early blood levels are considerably enhanced when the drug is taken in the fasting state. Five hours after a single dose, however, there are few differences in blood level determinations, whether the dose was given in the fasting or nonfasting state [5].

Blood and urine concentrations of phenethicillin potassium indicate that absorption from the alimentary tract is much greater than with penicillin G and twice that of penicillin V in the first hour [1]. The serum protein affinity of potassium phenethicillin is approximately equal to that of penicillin V [6]. Minimal inhibitory concentrations and CD_{50} values covering a wide variety of organisms show that phenethicillin does not differ significantly from the other forms of penicillin in antibacterial activity [7]. In a pilot study, Osment [8] reported satisfactory results with the drug in 29 of 33 patients with skin infections, and Carter [9] obtained complete remission of mixed infections in 136 of 141 patients. An evaluation of the clinical usefulness and safety of phenethicillin was made in patients seen in private practice who had a variety of general bacterial infections due to penicillin-susceptible pathogens.

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*Maxipen, product of J. B. Roerig and Company, Div., Chas. Pfizer & Co., Inc. (formerly known generically as α-phenoxyethylpenicillin potassium).

METHODS OF STUDY

Twenty-five male and 34 female patients, ranging in age from 11 to 85 years, were treated with phenethicillin for various bacterial infections (Table 1). Infections were mixed and multiple in some patients. The period of treatment extended from 3 to 60 days, the average being 15 days.

Table 1. Infections* Treated with Oral Phenethicillin

Infection	Number of cases
Respiratory	
Pharyngitis (acute)	18
Pneumonia (acute)	4
Otitis media (acute)	3
Pneumonitis	2
Rheumatic fever	2
Sinusitis	1
Lobar pneumonia	1
Bronchiectasis	1
Staphylococcal pneumonia	1
Skin and soft tissue	
Pustular acne	7
Furunculosis	6
Carbuncles	2
Cellulitis	2
Decubital ulcers	1
Supporative parotidis (acute)	1
Mastoiditis	1
Abscesses	
Teeth	1
Breast	1
Buttocks	1
Axillary	1
Post incision	1
Gastrointestinal and miscellaneous	
Ulcerative colitis (severe)	1
Peritonitis	1
Cystitis	1
Osteomyelitis	1
Gonorrheal urethritis	2

*Multiple, in some instances.

Dosages differed somewhat with the age of the patient and the nature of the infection. Thirty patients received 250 mg of phenethicillin, q.i.d. Five patients received 250 mg, t.i.d.; 2 patients, 250 mg, q. 3 h.; and 2 patients, 250 mg, q. 4 h. Nineteen patients were given 500 mg, q.i.d., and 1 patient received 500 mg, q. 3 h. Adjunctive therapy in 29 patients in this series included the administration of succinylsulfathiazole, chloramphenicol, kanamycin, streptomycin, erythromycin, and tetracycline, in addition to the oral penicillin and surgical and supportive measures where indicated. These included incision and drainage, postural drainage, oxygen, blood transfusion, tooth extraction, ileostomy, and tracheotomy.

Bacteriologic studies to identify the offending pathogen were done by the disc-susceptibility method. It was considered important to use discs in strengths of 10 μg/ml so that the in vitro active concentration of phenethicillin potassium necessary to inhibit growth of organisms with relatively high MIC values could be effectively determined.

Organisms isolated were Staphylococcus aureus, nonhemolytic; Staphylococcus albus, hemolytic and nonhemolytic; Streptococcus sp., hemolytic and nonhemolytic; Diplococcus pneumoniae, nonhemolytic; Streptococcus viridans; Bacterium coli; Vincent's spirochete; Haemophilus influenzae; and gonococcus. Testing of the organism by the disc method was carried out against penicillin, streptomycin, tetracycline, erythromycin, oleandomycin, novobiocin, chloramphenicol, and sulfonamides. Hematology studies done on patients before and after therapy included hemoglobin, white, and red blood cell counts.

Where the bacterial infection was brought under complete control, results were judged clinically excellent. If a definite remission of infection occurred without complete control, results were considered good. No change in the infection was termed poor results. Evaluated accordingly, 47 patients (80%) had excellent results; 6 (10%) had good results; and 6 (10%), poor. Four of the six failures occurred in patients experiencing side effects or sensitivity reactions necessitating discontinuance of therapy. The fifth failure occurred in a patient with acute pharyngitis due to a hemolytic streptococcus; when remission was slow to occur with penicillin, the patient was given another antibiotic. The drug also failed to produce results in a patient with an acute cystitis due to B. coli.

The only changes seen in blood were the expected decrease in leucocytosis as the infection was brought under control.

Good correlation between in vitro studies and in vivo results was shown, probably as a result of using the disc in 10 μg/ml strength. Pathogens were seen to be susceptible to penicillin in about 90% of the cultures, the same ratio of patients in whom excellent and good results were obtained.

SIDE EFFECTS

Minor side effects consisting of mild diarrhea in three patients and mild nausea in two patients did not necessitate discontinuance of therapy. Acute fungus infection of mouth, tongue, and gums occurred in one patient who had previously experienced the same side effect with broad-spectrum antibiotics; treatment with penicillin was not discontinued. One patient, already experiencing diarrhea from severe ulcerative colitis, found that this was intensified by oral penicillin, and the drug was therefore withdrawn. Sensitivity reactions occurred in three patients, one of whom had a past history of penicillin-sensitivity with the injectible form. This patient experienced acute urticaria within 15 min of ingesting the first trial 125-mg dose. The second patient developed urticaria on the fifth day of therapy. The third patient had an asthmatic (allergic) attack following 3 doses of oral penicillin. When the drug was withdrawn and appropriate measures taken, all three patients had uneventful recoveries. On the other hand, three additional patients with prior histories of allergic reactions to intramuscular penicillin experienced no side effects or untoward reactions to the oral drug.

CONCLUSIONS

Despite the obvious advantages of the oral route, it may be tentatively stated that the usual precautions regarding immediate or delayed allergic reactions to penicillin must be observed. The excellent and good results obtained in 53 of 59 patients (90%) given oral penicillin (potassium phenethicillin), with or without other medication and treatment for general bacterial infections, however, indicate the use of this form of penicillin in the treatment of infections caused by susceptible organisms whenever oral therapy is feasible.

The high blood levels promptly attained with phenethicillin give assurance of consistently dependable therapeutic responses formerly obtainable only with injectible penicillin. Phenethicillin is efficiently absorbed from the alimentary tract and yields high blood concentrations with an antibacterial effectiveness per unit equal to those of other forms of penicillin. The present drug fills an important therapeutic need and will doubtlessly take its deserved place as a standard medication in the existing antibiotic armamentarium.

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REFLECTIONS ON THE NECESSITY OF CONTINUING RESEARCH FOR EFFECTIVE ANTIFUNGAL AGENTS

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It is becoming increasingly apparent that there is a continually expanding field for the use of antifungal agents in the therapy of human disease. Serologic studies in tuberculosis sanatoria, carried on by the Public Health Service, now indicate that at least 8000 presumptive cases are admitted yearly. If proper laboratory studies are completed on these patients, at least one third of these presumptive cases can be culturally proven. Cases have also been demonstrated in Canadian sanatoria. Most cases are of the chronic pulmonary type requiring prolonged treatment in a manner similar to tuberculosis. The numbers of acute pulmonary histoplasmosis and disseminated histoplasmosis requiring treatment are difficult to estimate, but these numbers are substantial since evidence indicates that 500,000 people a year are infected with this fungus. Every year increasing numbers of reports of both the desseminated and the acute pneumonic type of histoplasmosis are appearing.

Coccidioidomycosis is widely prevalent in the Southwest and an estimated 100,000 people a year are infected, not including visitors. It has been estimated that one third of these develop severe enough illness to require visits to a physician. Chronic coccidioidomycosis, either pulmonary or disseminated, occurs with considerable frequency.

Infections with blastomycosis and cryptococcosis are increasing in frequency and importance as causes of pulmonary disease.

In addition, visits to South America have revealed that histoplasmosis and South American blastomycosis occur in the three major river basins of this continent. It is quite apparent that thousands of cases of active histoplasmosis and South American blastomycosis may be found in South America. In Sao Paulo, Brazil alone, records of more than 900 cases of South American blastomycosis have been accumulated. It is also evident that coccidioidomycosis exists in Argentina and Venezuela, and possibly in Brazil. It is quite clear also that the prevalence of mycotic infections is just beginning to be appreciated in many other parts of the world.

When one considers that the presently available antifungal agent, amphotericin B, is cumbersome to use and accompanied by undesirable side reactions in almost every case, it is apparent that there is a pressing need for the development of effective oral antifungal agents. Due to the difficulty in administering the drug and the fact that it must be given while the patient is hospitalized, it is presently employed only as a life-saving measure in acute, severe histoplasmosis, disseminated disease, or chronic, advancing cavitary disease. Hundreds, indeed, thousands of milder cases would benefit by the use of a less cumbersome antifungal agent were such an agent available.

PANEL DISCUSSION
on
CLINICAL PROBLEMS

October 28, 1960 1:00-3:00 P.M.

MODERATOR: DR. KIRBY

PANEL: Dr. Sidney Ross, Washington, D. C.; Dr. A. Bondi, Philadelphia, Pennsylvania; Dr. M. Romansky, Washington, D. C.; Dr. P. Bunn, Syracuse, New York; Dr. J. Sylvester, Chicago, Illinois

The following subjects were open to discussion:

1. Staphylococcal infections:
   a. Optimal therapy of severe infections.
   b. Management of nasal carriers.
   c. Furunculosis—treatment and prevention of recurrences.
2. Summary of the clinical potential of new antibiotics described at this meeting.
3. Potential role of new antibiotics about to be released for general use:
   a. Dimethoxyphenylpenicillin.
   b. Colistin.
4. Clinical applications and relative merits of the oral penicillins.
5. Clinical significance of bactericidal versus bacteriostatic antibiotics.
6. Clinical usefulness of currently available antibiotic susceptibility tests.
7. Antibiotic blood levels—clinical meaning.

DR. ROMANSKY: Dr. Kirby, I think we have to divide the therapy of the severe infections into basically two groups. First, those microorganisms which are basically responsive to current penicillin, exclusive of the dimethoxyphenylpenicillin, and those that are resistant to penicillin. In those which are responsive to penicillin generally, all other therapy being equal, such as evacuation of necrotic or pustular foci, as far as the chemotherapy alone of the penicillin-sensitive ones is concerned I believe, one would use large amounts of soluble crystalline penicillin. In those that are known to be resistant, or in those infections in which we do not as yet know what the sensitivity test will reveal, our current procedure, which may or may not be different from others, is to use ristocetin or vancomycin, holding as our third choice kanamycin. In the actual dosage of the agents, we use generally a gram at 8-hr intervals the first few days. It is particularly important that these two agents be given in a relatively small volume of solution rapidly withint 10-15 min for two reasons; one, to minimize the thrombophlebitis, and secondly, to produce a high concentration for maximum bactericidal action. Once the organism's sensitivity to the various antibiotics is known, if it turns out to be responsive to penicillin, one might switch to it, although in our situation, if the patient is doing well, we continue to use either ristocetin or vancomycin.

Now, the third area is the question of the dimethoxyphenylpenicillin and its effect upon what should be done. I don't think at this moment we can say that we can automatically turn to the use of dimethoxyphenylpenicillin, but it certainly opens the avenue to its use in resistant staphylococcal infections.

DR. KIRBY: Thank you, Dr. Romansky. Dr. Romansky would use either ristocetin or vancomycin, giving a gram every eight hours and injecting it in about 10 or 15 min in 200 ml of fluid. Let's ask Dr. Bunn if he would agree with this or if he would do anything differently about it.

DR. BUNN: We have altered our direction in the last 6 months and what I would choose is to put the dimethoxyphenylpenicillin first on the list as the drug of choice. It has some singular advantages although our experience with it is limited to a reasonably few cases. I think because of its ease of administration, and its lack of toxicity, that by all odds right now it is our drug of choice, rather than vancomycin or ristocetin.

DR. KIRBY: Do you have any evidence, Dr. Bunn, that it is better than vancomycin or ristocetin?

DR. BUNN: Of course, you can measure activity in a number of ways. I suppose mortality rate is the easiest. It is generally conceded that those who have a life-threatening staphylococcal disease and a positive blood culture would, without any treatment at all, die in 80% of occasions. One can take it from that point on. In the recent summary of about 200 seriously ill patients with staphylococcal disease, it would appear that the mortality rate had fallen to about 20% and perhaps to less than 10%, depending upon your criteria. I think this is a rather remarkable figure, although I would hate to try to compare that figure with any other single drug. Incidentally, infections caused by the few organisms that did remain susceptible to penicillin G did remain the easiest to treat and gave the best results.

DR. KIRBY: You would agree, wouldn't you Dr. Bunn, that these 200 cases treated with Staphcillin® in which the mortality rate was under 20% are not necessarily any different than certain series with, say, vancomycin, where the mortality rate was 50 or 60%? Our figures are not really adequate to say Staphcillin has lowered the mortality rate, as compared with vancomycin, from 50%.

DR. BUNN: I don't think so, Dr. Kirby. I don't think that experience is broad enough to make that clear-cut statement at this time. I sure hope it is true and I hope it will stay up for the sake of patients.

DR. KIRBY: Let's see what Dr. Ross has to say about serious staphylococcal infections in children, where we have a problem certainly of administration.

DR. ROSS: I would agree with Dr. Bunn. Because of the difficulty and technical problems of administration of ristocetin and vancomycin in the pediatric age group, we would certainly be in favor of the use of Staphcillin in severe infections. I mentioned yesterday at the meeting here that the last two children that had been treated with vancomycin, one with an acute osteomyelitis, the other with SBE and associated staph meningitis, posed such problems attendant to the administration of vancomycin that we finally resorted to the cut-down method, instilling a polyethelene catheter right into the inferior vena cava and with that modality of administration we were able to continue the drug for 30-40 days. I would say certainly in the pediatric age group that Staphcillin would be immeasurably more readily administered with considerably fewer technical problems.

I want to call attention to one thing in passing. By and large the children that we have encountered who have picked up staph infection on the outside have an organism that is susceptible to penicillin G. However those who picked up their infection in the hospital usually are resistant to penicillin G and would require one of the other drugs. I want to mention also that it is important in the treatment of acute osteomyelitis in pediatric age groups to continue the therapy for a sufficient period of time. Now, what constitutes a sufficient period of time? It's arbitrary, but certainly it is four to six weeks—that would be a desirable hiatus.

DR. KIRBY: I think the point Dr. Ross made about the tolerability is certainly important. The Staphcillin can be injected intramuscularly and is quite well tolerated. One fourteen-year-old boy we treated for osteomyelitis of the hip received the Staphcillin into the other buttock every four hours for 20 days and tolerated it extremely well. This is an example of the fact that this can be done and this certainly makes the problem much simpler in children. Another thing that we might mention briefly is that many pediatricians have reverted to bacitracin in treating small babies. There are now enthusiasts who are going back to giving bacitracin intramuscularly. My own reaction to this is that this can be done, but that the blood levels attainable with bacitracin are simply not comparable to those one can get with vancomycin or with Staphcillin. Bacitracin is a bactericidal drug but blood levels are in the range of 2 or 3 μg instead of the 15-20 that one can get readily with Staphcillin and 30-40 that one gets readily with a gram of vancomycin or ristocetin. I was just wondering if Dr. Ross would care to comment on the use of bacitracin and whether he thinks it has any place in therapy now with these other drugs available.

DR. ROSS: Dr. Kirby, I do not think so. With the advent of relatively safer antistaph agents, I feel that bacitracin has only historical importance. Let me just mention one oblique note on the use of dimethoxyphenylpenicillin in the pediatric age group. Currently we're employing 100 mg/kg for q 6 hours divided dose schedule for the average case. We have even gone up as high as 200 mg/kg.

DR. SYLVESTER: I am sure that Dr. Kirby had this thought in mind when he invited Dr. Bondi and me to participate in this panel and that is in the area of the significance of laboratory information. I would like to stress in this area one or two points that have not been brought out yet. I think clinicians like Dr. Kirby and Dr. Romansky make the assumption that they have good laboratory data available on which to base their choice of the antibiotic providing optimal therapy. I think that this is a very important factor in the selection of the antibiotic, that laboratory information be available to indicate what the optimum therapy is in terms of the agent selected. Particularly in small hospitals, under 150 beds, where the laboratory facilities are not always good, it is sometimes a problem for the physician to obtain dependable sensitivity data on which to base his decision as to the agent. The next point in this same regard, I think, is the question of not only the right agent but the right dosage, and that is an important factor. And the third point is one that Dr. Ross has already touched upon and that is in severe staphylococcal infections, particularly when they might be considered life-threatening, the physician might have to make an assumption as to the sensitivity of an organism and this assumption may or may not always be right. But in many instances such assumptions will be helpful in the initial choice of an antibiotic, and the management of the patient can be changed when adequate laboratory data are available. But I think inherent in choosing optimal therapy is adequate microbiological information as to the sensitivity.

DR. KIRBY: Dr. Bondi, do you have any points here?

DR. BONDI: I have been criticized at this meeting by several of the individuals interested in the problem of sensitivity testing for not taking a definite stand about methods. In this respect, I feel a little bit like the boy who, after killing his father and mother, throws himself on the mercy of the court as an orphan. By this I mean that although I was one of those who helped get the disc test going, I have not been very active in this field in supporting it. We still use this test. I think it's very valuable, and I think very definitely we still need people like Russ Fowler and Ted Anderson to be constantly reminding us of the pitfalls of the method and to spread this gospel around. I am not worried about the laboratory testing in the laboratories of the people represented at this meeting. I am concerned about the many laboratories in the country which are run by people who really don't know what they are doing. I feel that any method of sensitivity testing is good if it's run by competent people, but I am afraid I'll have to admit that too often such tests are being run in laboratories by individuals who really are not competent to evaluate the method that they are using.

DR. KIRBY: Thank you, Dr. Bondi. I think it's appropriate for Dr. Bondi, who is one of the originators of the single-disc method of doing sensitivity testing, to make this comment, because he did this work a good many years ago. It certainly is true that as the disc method is now used and practiced that it is common for laboratories, for example, to give the report of vancomycin as slightly active with staphylococci because it has not diffused well and does not give large zones. This is just one illustration of the fact that adequate standards for zone sizes are not available and are not promulgated by the people who are doing the disc sensitivity tests. I think we can say that for severe staphylococcal infections the bactericidal drugs vancomycin and Staphcillin are, at the moment, probably the agents of choice. The potential of Staphcillin is now known. We want to emphasize this. The preliminary results look extremely good, perhaps comparable with those of vancomycin, but more study will be needed. Staphcillin needs to be injected every four hours intramuscularly, which is something of a problem, but in many individuals this is less of a problem than intravenous administration every eight or twelve hours. Now, does anyone from the audience want to disagree violently, or ask a question, or make a point about severe staphylococcal infections?

Q: Has Dr. Bunn switched from kanamycin to Staphcillin?

DR. BUNN: Yes, we have.

Q: How about kanamycin in children. Some people have had excellent results in children with kanamycin.

DR. KIRBY: Here again, I think we have the problem of ease of administration. How does Dr. Ross now compare kanamycin with these other three drugs?

DR. ROSS: We have had a moderate degree of experience with kanamycin in pediatrics. I would say parenthetically that because of the potential toxicity of this drug, I would much prefer to use Staphcillin and perhaps to a lesser degree vancomycin or ristocetin.

DR. KIRBY: A question from the audience, as I understand it, is: Should Staphcillin be reserved for known severe staphylococcal infections, or should it be used in a fairly haphazard fashion upon suspicion of infection? I will give my own views on this. I am one of those who do not especially anticipate that resistance will necessarily develop to Staphcillin. The good reason for saying that is that staphylococci have not become resistant to penicillin G. The only staphylococci resistant to penicillin G are those which produce penicillinase. In the test tube, it's true, you can make staphylococci resistant to penicillin G, as well as to Staphcillin, but it's at about the same rate as with penicillin G and I think there is reason to be optimistic on this point. I don't think that at the moment there is any reason to say that this is a drug that should be used rarely. I think in a patient who may have a severe staphylococcal infection one now has the alternative of using a drug like vancomycin or Staphcillin, and one might prefer vancomycin because I have seen many patients who would rather have a single injection of a gram of something every twelve hours than to have an intramuscular injection every four hours. Others would prefer it the other way. I would like to ask if any other panel members would feel differently about this and would feel that this new penicillin should be reserved?

DR. BONDI: I see no reason to withhold the use of dimethoxyphenylpenicillin at all for this fear of development of resistance. Secondly, I think the question as it was posed is one which can be answered by personal clinical judgment. And because I would do one thing and Dr. Kirby would do another, I don't think that makes either of us necessarily right or wrong. In my own personal opinion, I would have no objection whatsoever, in a seriously ill patient in whom the judgment was that he had a staphylococcal disease of serious proportion, to start right off without adequate laboratory isolation of that organism with dimethoxyphenylpenicillin and, indeed, this is precisely what we do.

DR. KIRBY: There is a question back there. Does Benemid improve the levels of Staphcillin? Yes, it does. Dr. Romansky.

DR. ROMANSKY: I would just raise one caution that we raised the other morning and that is this, it does not lessen the need for a careful history as to whether or not a patient is allergic to penicillin. And I would answer this questioner's question in this fashion. Assuming that you are sure that the patient is not allergic to penicillin, my own personal feeling at this moment, until more information is available, still is that I would use one of the two agents I mentioned, either ristocetin or vancomycin, as of this moment, as my first choice for patients severely ill who appear to have a staphylococcal septicemia.

DR. KIRBY: Now, Dr. Romansky says he is worried about whether this patient is allergic to penicillin. I would like to ask Dr. Romansky, who also presented a paper here on pneumococcal meningitis, if he bothers to ask the unconscious patient with pneumococcal meningitis whether he is allergic to penicillin. We don't and I just wondered if he does.

DR. ROMANSKY: We do, for a very good reason. You unfortunately missed the conference when we discussed this. We unfortunately had a tragic problem, which I think can occur again. We did have a woman in her middle forties who came in with pneumococcal meningitis, severely ill, and was given two million units of penicillin intramuscularly on the first dose and two hours later was begun on intravenous penicillin, because she began to go into a shocklike state. Within a fraction of a few moments after the first administration of the intravenous penicillin, she developed a progressive angioneurotic edema beginning at her feet going up to her head, and then went into anaphylactic shock. Fortunately, she came out of this shock, but two hours later, again went into anaphylactic shock and expired, and I can only tell you that, as a matter of fact, for some time now, we have had a ruling on our service that patients unable to give an adequate history because of coma or who do not speak the language will not get penicillin. I would also point out, as we did the other morning in our discussion, that one is not limited by penicillin. In pneumococcal or meningicoccal meningitis, if one is willing to give an adequate dose of the other agents, be they erythromycin or tetracycline, or chloramphenicol, in doses of 4-8 g intravenously, results will be just as adequate.

DR. ROSS: In that regard, the difference in the incidence of allergenicity in the adult group, as opposed to the pediatric age group, is that about 5-8% perhaps of adults are allergic to penicillin but in pediatric age groups only about 0.5% are. Thus we don't run into this problem very frequently and hence we don't generally have too many reservations about the administration of penicillin in children.

DR. KIRBY: Dr. Bunn wanted to make a point about this allergic tendency in relation to Staphcillin.

DR. BUNN: From preliminary observation it would appear that Staphcillin, although I am sure a patient could get allergic to it, is immunologically distinct. A patient with a rash or a fever or anaphylaxis from penicillin G can tolerate without a continuation of that reaction the administration of dimethoxyphenylpenicillin. In other words, I think that the history of a reaction to penicillin G should not interfere with the administration of the newer synthetic materials, because they will not cause the same kind of reaction.

DR. KIRBY: You think that no one getting his initial shot of Staphcillin is going to roll over dead from this drug.

DR. BUNN: I don't think that can be answered very fairly. It hasn't happened yet. It is feasible that it could happen; it does with patients who are allergic to penicillin G on first exposure. I don't think the incidence of reactions to dimethoxyphenylpenicillin is going to be any different than it is to G except that one does not enhance the development of the other. They are distinct drugs. It would be the same as checking insulin, for instance, against penicillin.

DR. ROMANSKY: Just one point. We should remember that the first anaphylactoid fatality was not reported until 1946, despite the fact that we began using penicillin in 1942 and 1943, and in 1946 there were 356 reports with a 9% mortality. I raise this question for the very reason that there are the two hundred cases Dr. Bunn reported. I think this is a relatively small number, but I would still emphasize the point that one should be extremely cautious with unconscious patients who are unable to give an adequate history.

DR. KIRBY: The question is raised in the audience regarding duration of therapy in adults. I would like to answer and comment on that myself. Here again the duration is getting shorter and shorter. In the 33 patients with staphylococcal septicemia that we treated with vancomycin, of the 20 cured, 12 received only two weeks of therapy. And, in general, I would say that our impression and tendency in treating staphylococcal sepsis is that treatment for four to six weeks is rarely necessary; often two weeks is adequate, and for most cases three is plenty. The trouble, of course, is that you never know what you are treating. I am sure that a patient with a cut-down infection, once you have removed the cut-down, is an entirely different thing than a patient who has been ill for months and has a huge vegetation growing on his heart valve; so I don't think one can really generalize, but I would state as a generality only two to three weeks rather than four to six weeks for severe staphylococcal infections, but I think this is a more clinical hunch than it is something that I can document very well for you. Let's see what the others have to say about that. Dr. Bunn.

DR. BUNN: Oh, I agree. I don't think you can ever make any general statements about what kind of program is in order. The patient's disease makes a tremendous difference. I would be very much more inclined to treat vegetative disease, like endocarditis, for a longer period than I would for, say, a carbuncle, which could be treated also with the help of a surgeon. For an acute osteomyelitis, for example, I think the treatment has to persist for longer periods of time. In other words, the judgment of what you think has happened to the body tissue determines the duration of therapy. As far as concerns the length of time for using Staphcillin, there isn't any reason it can't be used four or six or eight weeks without difficulty, although I agree heartily with Dr. Kirby, that we don't use it that long very often because it is not necessary.

DR. KIRBY: I would just like to say that the person who asked this question, Dr. Fremont Smith, is as much of an expert on this subject as anyone in the room, and I would like to ask his opinion.

[COMMENT FROM THE FLOOR (Dr. Fremont Smith): Transcription unintelligible.]

DR. KIRBY: I certainly think Dr. Fremont Smith has brought out a very important point. There are the patients who, following treatment for staphylococcal septicemia, go on getting one seeded staphylococcal abcess after another and if this persists, often they die of it in two or three years. The problem is, should we treat everyone for six or eight weeks to obliterate this mistake? I'm not convinced that in treating anyone for six or eight weeks we are preventing this error and this is where the problem lies.

DR. ROMANSKY: I think, Dr. Smith, we can divide these, perhaps on the basis of some preliminary observations we have made, into groups of septicemia and those with perhaps staphylococcal endocarditis. As you probably know, in one of the first papers on erythromycin, we thought the possibility existed to treat staphylococcal endocarditis for a couple of weeks as we had our enterococcal group. At this time, my opinion has changed completely, based on the following: We have studied very carefully the patients with staphylococcal endocarditis who received either vancomycin or erythromycin and at the end of three weeks, we stopped treatment and then did renal biopsies. At the time we did the renal biopsies, the blood cultures were negative, that is, they were still in the process of being maintained on treatment when cultured. The renal biopsies were very interesting, because each one of them showed active necrotic foci with plenty of staphylococci, intimating the possibility that although we had perhaps reversed the lesion on the valve, we had a good source for seeding. It so happened that in both of these instances the cultures ultimately became positive. So, at the present time, with both erythromycin and vancomycin, we continue our treatment for no less than six consecutive weeks and I am fairly convinced that anything less than this in endocarditis may be a very hazardous thing, particularly in relation to the point you make about embolic phenomena.

DR. KIRBY: All right, I think we will terminate the discussion of this subject at this point. There is, obviously, a short-term group and a long-term group here. Now, I think I'm going to use the chairman's prerogative of skipping down our list of subjects to another one. We will return to the problem of nasal carriers in due time, but I would like to get into another problem, if we can. Namely, I would like to hear some discussion of the clinical applications and relative merits of the oral penicillins. There has been quite a battle of the blood levels going on and I think we should have some frank discussion here about the relative merits of these agents. Specifically, I would like to hear what people think about the advantages, if any, of the semisynthetic penicillin, α-phenoxyethylpenicillin, over penicillin V. There are strong proponents on both sides and there are those who feel that these drugs are, for all practical purposes, interchangeable. I would like to hear an expression of opinion on this. Let's ask Dr. Bunn.

DR. BUNN: For potency, ease of administration, and expense, these two drugs are very much alike. The one area where it seems to me that perhaps there are distinctions is in case of allergies. It would appear that the individual allergic to penicillin G will similarly be allergic to penicillin V, but not to a phenethicillin. In this area, at least for the moment, it has some singular advantage, but only in this very restricted group where an individual is allergic to penicillin G or penicillin V. As far as the potency is concerned, I would use them interchangeably, both in the dosage, duration of the therapy, and in the indications for the use of an oral preparation.

DR. KIRBY: Dr. Bunn, did you say now that patients who are allergic to G or V are not allergic to phenethicillin?

DR. BUNN: That is correct. These are immunologically distinct.

DR. KIRBY: Do we have adequate clinical evidence on the differentiation in this respect?

DR. BUNN: Well, I would hate to recite anything more than just personal experience, but we have had that experience, I suppose, more than a dozen times now.

DR. KIRBY: Do a dozen experiences mean anything, though, when it is presumably true that if you have a reaction with penicillin V you may never have another? In other words, is this not such an unreproducible phenomenon that the 12 cases are not adequate?

DR. BUNN: Well, all of them, I guess, had rash when G was stopped and phenethicillin was substituted while the rash was still present, and the rash would disappear. I think this is more than just a coincidence.

DR. KIRBY: Then you don't think large-scale studies are necessary to establish this point?

DR. BUNN: I think large-scale studies are very difficult to set up on this point. It would be awfully nice if somebody had such a large group of individuals with known allergies to G to really prove it. As I said, this is a personal experience and it doesn't cause me any fear to continue the second drug when they are allergic to the first. Now whether or not this is a proven point, Dr. Kirby, I would be the last to defend it completely. It is our original impression, that it can be used in that kind of situation.

DR. KIRBY: All right, does anyone else care to comment on this?

DR. GRIFFITH: Dr. Kirby, I think that it is the experience of most of us who treat patients that we have seen patients receiving penicillin V develop allergic reactions and because we have nothing else to use, continue penicillin and see it disappear. This so-called desensitization effect is described in the literature. One of the reasons we have gotten into trouble by continuing penicillin in patients who manifest an allergy is that the majority will go on and develop a more severe reaction. However, there are those who will be desensitized. We have also had this experience. Just recently we had a patient who had a history of a year previously having had a penicillin reaction with dermatitis. She spent two weeks in the hospital with it, and was then admitted to our hospital ward and received procaine penicillin, the same material which she had received before, and she took it without any difficulty. So, I think when you have patients to whom you give a new penicillin and they do not develop a reaction, you can't conclude that this means this penicillin is less allergenic. I do think it means you should test these individuals with the material you think they were allergic to before, such as penicillin G; if they are allergic to that, then go ahead and give your new penicillin. If you have nerve to do that, then I'll conclude that you are right.

DR. KIRBY: All right, Dr. Griffith has some question as to whether Dr. Bunn's cases wouldn't have done just as well if he had kept them on penicillin G. How about that, Dr. Bunn?

DR. BUNN: I have no comment about it. Dr. Griffith's observations are perfectly correct, and I am sure we've had that experience too, seeing patients that have been allergic to penicillin G subsequently being able to tolerate it.

DR. KIRBY: Dr. Bunn, what do we mean when we talk about an allergic reaction to penicillin?

DR. BUNN: Well, without going into a lecture on hypersensitivity reactions, it is surely true that when one gives a drug like penicillin there are three different kinds of things that can happen. The first, and most abrupt and acute, is acute anaphylaxis with some shock organ, generally the lung. There are individuals with delayed anaphylaxis or delayed reactions of one order or another which may appear several hours or a few days after the first exposure to the drug. Then there are those also of the third category, who develop rashes or penicillin dermatitis, if you will. These are all antigen-antibody reactions of one order, presumably caused by penicillin's ability to bind with the serum albumen when it is administered to the human, thus acting as a hapten in the development of an allergic phenomenon. It is no different than any other allergy.

DR. KIRBY: Dr. Romansky, would you care to enter into this somewhat divergent problem of whether phenethicillin is any different?

DR. ROMANSKY: I'll just confine my remarks to the biological and pharmacological activities. I think that there isn't a great deal of difference in the actual therapy of a patient with penicillin V or the new agents. When one actually looks at the serum levels the difference is not, in my opinion, significant biologically. With respect to penicillin G and sensitization, this point was very common in our early days at Walter Reed, giving penicillin where it was the only agent we had. Patients would receive it for a month, have signs of allergy, and a month or two later we would have to give it again because of the nature of the lesions. They were then able to tolerate it. So I would agree with Dr. Griffith, this is not uncommon.

DR. GOLD: In our series of 100 cases that we reported on with α-phenoxyethylpenicillin, we had no allergic skin reaction. We used it in nine patients who had previous history of skin reaction to procaine penicillin. We now have six patients who were on prophylactic penicillin for rheumatic heart disease, who developed skin allergy to penicillin G tablets. These patients, all six of whom are on a follow-up now of approximately six months are on oral phenoxyethylpenicillin without any reaction. However, on the other side of the coin, our allergist showed me two cases who were allergic to penicillin G by history, in whom skin reaction occurred to α-phenoxyethylpenicillin as well.

DR. KIRBY: Thank you, Dr. Gold. Are you from Syracuse or from Brooklyn? This is Dr. Gold from the Downstate Medical Center in Brooklyn. All right, any other comments on the problem of allergy? Dr. Perrin Long would like to make a comment on this while we are at it.

DR. LONG: If you fellows want to solve this, get some asthmatics and give them penicillin!

DR. KIRBY: Dr. Long says if we want to solve this, get some asthmatics and do a cross-over study—is that the idea? We would have to have a little adrenalin on hand, wouldn't we?

Now, I would like to see if we can clarify this matter of blood levels a little bit. As I understand it, with the α-phenoxyethylpenicillin the microgram blood levels seem to be a little higher. On the other hand, it may be that α-phenoxyethylpenicillin is a little less active against streptococcus pneumococci and susceptible staphylococci so that the microgram advantage is just about offset by the difference in activity. If this is true the sum totals of the blood levels would, for all practical purposes, be the same. Dr. Bunn gave us a talk yesterday to the effect that blood levels don't mean much anyway because of the way they are done. Therefore, I would like to ask Dr. Bunn if he would agree with this. Are the blood levels for all practical purposes identical between these two oral penicillins?

DR. BUNN: No, I don't think they are identical in the way we generally measure them. As a matter of fact, in the laboratory, depending upon the technique used, one can manipulate it so that penicillin V level is higher than phenethicillin level, or vice versa. I agree, as Dr. Romansky said a moment ago, that there are not significant variations to the point that one would be willing to alter a dosage program. They are sufficiently alike, it seems to me, that their dosage ought to be essentially the same. My comments yesterday in the paper were to point out that the comparison between the levels of the two penicillins can be manipulated by use of dosages and the use of the test organisms to come out with almost any answer you wish.

DR. KIRBY: What we want to know is, what is the right answer? Is there a right answer?

DR. BUNN: I don't think there is any right answer available from the blood level work which we have had described in very many places by very many different laboratories. I don't think the answer can be secured from the kind of information that we have had available so far.

DR. KIRBY: Do you think there are criticisms that can be made of all the blood level studies for strict comparative purposes?

DR. BUNN: Yes, I'm not being critical of the work which has been done on it. I am somewhat critical of the comparison work.

DR. KIRBY: Would you though, in actual practice, if you now are treating a patient with bacterial endocarditis and you want to send him home after a week in the hospital to finish his course of therapy at home, as we have been doing, would you just as soon use either one of these two forms of oral penicillin?

DR. BUNN: I would use them interchangeably at the same dose level.

DR. KIRBY: All right, Dr. Romansky, would you?

DR. ROMANSKY: I would use them interchangeably so that a patient from an economic standpoint, would get the cheapest. I think that is the important thing.

DR. KIRBY: As I understand, there is no difference here, is there?

DR. ROMANSKY: I don't know.

DR. KIRBY: I believe that is right. Does anyone else care to comment? Somebody says that penicillin V is 15% cheaper. Dr. Ross?

DR. ROSS: I would like to ask Dr. Kirby one thing. Let's take the Staphylococcus. In comparing blood levels with antibacterial activity, and that seems to be a popular sport at the moment since staph is either quite sensitive to G or V or it is quite resistant, would it make a great deal of difference if it could be shown that the antibacterial activities of V were somewhat higher than to semisynthetic penicillins?

DR. KIRBY: I don't get the question. Would you repeat it?

DR. ROSS: I wondered if any mild or moderate differences in antibacterial activities would make a great deal of difference since staph is either quite sensitive or very resistant.

DR. KIRBY: Yes, I have tried to avoid staphylococcal infection. What we are talking about here is comparison of blood levels against sensitive organisms. And we are talking chiefly, I think, about streptococci, pneumococci and sensitive staphylococci. Would we choose one of these drugs over the other on the basis of the existing blood level data? I won't get into the staphylococcal part. Dr. Ross, does that answer your question? I think Dr. Sylvester had something to say about this.

DR. SYLVESTER: My comment is not on this particular question, but I would like to interject into the discussion at this point that there are many factors involved in the efficacy of a chemotherapeutic agent in the host besides its concentrations and the specific in vitro activity of that organism. Some few years ago we reported at one of these meetings a comparison of the in vitro and in vivo effectiveness and tried to quantitate this relationship. It was very obvious with the four antibiotics that we studied that there was a tremendous difference in the actual effectiveness of these agents in the host, more than you could expect from their in vitro activity. The point that I'm trying to make is that I think that some of the concern about blood levels borders on the ridiculous because, in reality, this is not a complete measurement of the therapeutic efficacy of the antibiotic or the agent in the host. There are so many other factors that I think are involved that are probably more important.

DR. KIRBY: Well, but this is all we have, isn't it? We know the in vitro activity and we know the blood levels. And this is all we have to go on in making our judgments when we have two apparently close medications to choose from. What else have we to go on, Dr. Sylvester? I think we would be happy to have something else, if you can tell us what it is.

DR. SYLVESTER: What I am suggesting is that minor differences in this particular point are probably not of enough importance to be concerned about from the therapeutic standpoint.

DR. KIRBY: Yes. I think this is the point. We are trying to see if we have agreement or disagreement on whether these are significant differences among the oral penicillins. All right, now, one final point about the oral penicillins, is that of staphylococcal infections. Should staphylococcal infections be treated with phenethicillin? Let's ask Dr. Romansky about that.

DR. ROMANSKY: Well, in a sense we are back where we started. In my opinion the basic principle holds, that until I know what the status of the microorganism is, I am not going to use any form of penicillin. We can't give the dimethoxyphenylpenicillin orally at the present time, so I would not use the oral penicillins at all for the minor staphylococcal infections or any other type until I knew a little bit more about the status of the organisms.

DR. KIRBY: And if it were a penicillinase producer?

DR. ROMANSKY: No, I wouldn't use it.

DR. KIRBY: Dr. Romansky says that he would not use any of the available oral penicillins in treating infections due to any penicillinase producers. Is this correct, Dr. Romansky? Dr. Bunn, would you agree with this?

Another question I would like to ask about oral penicillin is, are the new penicillins worth the difference in price? When they first came out, many people maintained that if you really use penicillin G by mouth and just give a little bit more, that this saves the patient a tremendous amount of expense. Despite this point of view there has been a tremendous swing toward oral penicillins in the last two or three years and to the use now of the potassium penicillin G and phenethicillin. Dr. Bunn, how about that; do you use the oral penicillin G at all? Which is cheaper?

DR. BUNN: I have a lot of friends in the audience, Dr. Kirby, but I must admit that we still prescribe penicillin G most of the time by mouth. We do two things; we up the dose to one-half million units, and we keep it away from meals, so that a program of three times a day would be 10 o'clock in the morning, 3 o'clock in the afternoon, and bedtime at night.

DR. KIRBY: Now, Dr. Bunn, would you do this with a patient where it really counts? I think the most dramatic one at the moment is a patient who formerly would have been in the hospital for four to six weeks with bacterial endocarditis. You can now send him home in a week on oral penicillin. Do you trust penicillin G for that purpose?

DR. BUNN: Sure.

DR. KIRBY: I, personally, would not. I think that the much more reliable blood levels with or without food favor the newer forms, since you can't guarantee the patients are going to take their medicines without food. I personally have, after doing exactly what Dr. Bunn does for sometime, switched over to the other side of this picture. Let's hear what Dr. Romansky says.

DR. ROMANSKY: I agree with Dr. Kirby. We use very little G since the amount of G that one would give in bacterial endocarditis would cost about the same as penicillin V.

DR. KIRBY: What is that point, Dr. Grimes?

DR. GRIMES: I don't think you ought to say that the cost should enter into this. If you really believe that one preparation is better than another, I think it ought to be prescribed despite its cost.

DR. KIRBY: It seems to me that is contradicting what you said before.

DR. GRIMES: Not at all. I never said that I thought that G was superior to penicillin V or α-phenoxyethylpenicillin.

DR. KIRBY: The reason that I am mentioning this is that in the last couple of months we have had four such patients. In all four, we did measure blood levels in the hospital with and without food while on the oral penicillin and we were so impressed with the levels that this gave us assurance to send them home. We have not encountered any nonabsorbers, but if there are such nonabsorbers, it would certainly indicate the advisability of measuring serum levels in every patient before having the confidence to send them home and save on the hospital bill.

I think that in the enterococcal infection one might treat them with two weeks of penicillin and streptomycin in the hospital, or two weeks of vancomycin. More recently we have been treating them with just two weeks of vancomycin and stopping. One could, then, if one wanted to, send the patient home on a suppressive dose of oral penicillin. Now, regarding the treatment of very susceptible streptococci with penicillin and streptomycin, let me say that in the last few years the two-week regimens have combined fifteen million units a day of penicillin G with streptomycin. As an alternative to four to six weeks of injectable penicillin (and our usual plan has been to omit the streptomycin altogether) we give the longer course of four to six weeks, but give most of it by mouth. Does that answer the question?

Any other comments on this point?

[COMMENTS FROM THE FLOOR: Transcription unintelligible.]

DR. KIRBY: We have only treated about three cases of sensitive enterococcal endocarditis with vancomycin. We treated them for two weeks and they have been cured. Three cases are too few to mean anything because I think you could treat three cases with almost any dose of penicillin, perhaps, and have three cures. I would like to refer that to the others.

DR. ROMANSKY: As you probably know, we have treated a series of enterococcal endocarditis with Erythrocin with what I think are excellent results. Dr. Kirby is getting similar results with vancomycin. We have treated six patients in the same fashion, 28 injections over 14 days with sensitive green strep with similar results. I personally do not hold with the use of oral penicillin in enterococcal endocarditis because our experiences with enterococcal endocarditis are such that one might suppress both the infection in vivo and suppress the growth in the culture tube and end up, as the history of so many of these patients indicates, with the persisting infection. There is one patient at Walter Reed now who has been on a gram or more of oral penicillin for weeks and weeks because he has no veins left and no other way of giving injections, and he is doing well clinically. But his blood cultures persist in being positive for enterococci. It is very hazardous, in my opinion, to treat enterococcal endocarditis with oral penicillin. I don't think, Dr. Kirby, you meant you would treat only with oral penicillin; you're following vancomycin with penicillin.

DR. KIRBY: Yes, and even then, I said you might do that. I was speaking more about the susceptible enterococci. If there are no more questions here, I think we will go to the next point, a summary of the clinical potential of the new antibiotics described at this meeting.

About six or seven new antibiotics that I have never heard of before have been described at this meeting. I have seen all the papers, but I have not been able to talk to everyone involved. As I understand it, these antibiotics have some very interesting characteristics. For example, porfiromycin, as I understand it, is considered too toxic at the moment to be introduced into therapy as an antibiotic, although it might possibly be useful as an antitumor agent. To the best of my knowledge none of the new antibiotics described here shows promise of immediate clinical systemic usefulness. If I am wrong about that, would someone correct me. Is there someone here who could speak for any of these agents from the standpoint of these drugs being clinically useful right now?

[COMMENT FROM THE FLOOR (Dr. Savage): Transcription unintelligible.]

DR. KIRBY: Thank you Dr. Savage. Does anyone care to comment about any of the other agents mentioned here? All right, I think we will consider under the next point, then, briefly, the potential role of new antibiotics described at this meeting that are about to be released for general use. We have already had a great deal of comment on dimethoxyphenylpenicillin or Staphcillin and I don't think they need any more comment. The other drug, colistin, might be commented on briefly, because I am sure there are many here who did not hear the paper and discussion on it, and as the co-author of that paper, I will just say a sentence or two.

As I see it, colistin is very similar in its action to polymyxin B. We feel that it is considerably less toxic, and much better tolerated. It is possible, in adults, to give 75 mg every six hours with a minimum of discomfort and a minimum incidence of neurotoxicity and nephrotoxicity. In urinary tract infections, Pseudomonas strains are eradicated in a fair percentage of patients. In patients who are seriously ill with Pseudomonas infections, who have abcesses deep in the tissues or endocarditis, I think it is perhaps fair to say that this drug is barely adequate. Some cases may be saved but the nature of the infection that the patient has and the nature of the antibiotic with its low serum levels, are such that one cannot hope for cures in a large percentage of patients with Pseudomonas septicemia or indeed severe Pseudomonas infection in other parts of the body. I think this is a brief summary statement about colistin that perhaps everyone would not agree with, and I wonder if anyone has any comments or any additional remarks to make here. Dr. Bunn?

DR. BUNN: No, I don't have any. I'd like to ask a question. Are you restricting its use to Pseudomonas infections, Bill? Is that what you are implying?

DR. KIRBY: No, not entirely. We have had some patients with chronic pyelonephritis with other antibiotic-resistant organisms, primarily Aerobacter and a few others, that we have treated with this drug with eradications of infection in some instances. Dr. Ross?

DR. ROSS: We have had some experience with colistin in the pediatric age group in the past year and a half. I would agree completely with Dr. Kirby that it is considerably less toxic than is polymyxin. In answer to Dr. Bunn's question about restricting colistin to its use in pyocyanic infections, I would agree that probably this is its chief area of usefulness. We treated a group of UG tract infections, including pathogenic E. coli, some monilia and shigellosis and also a couple of typhoids. I must say that we were not terribly impressed with its efficacy in pathogenic E. coli, and in shigellosis it was less attractive than broad-spectrum antibiotics, including the tetracyclines and chloramphenicol, and in some places it seemed to be moderately disappointing. I do think, however, it does have its chief role in Pseudomonas infection.

DR. KIRBY: Thank you Dr. Ross. Any other points here?

[COMMENT FROM THE FLOOR (Dr. Savage): Transcription unintelligible.]

DR. KIRBY: To my knowledge we are not talking about the colistin methane sulfonate which has been the form clinically available for quite some time. Is that right Dr. Schwartz?

[COMMENT FROM THE FLOOR (Dr. Schwartz): Transcription unintelligible.]

DR. KIRBY: The sulfate will be available orally and the methane sulfonate for the intramuscular. Thank you.

DR. SCHWARTZ: The American experience has been that colistin is primarily dramatically effective against Pseudomonas infections, not only of the gastrointestinal tract and urinary tract but also in eye infections. The literature from France, Italy, and Japan indicates that this antibiotic has a broad spectrum in terms of Gram-negative organisms. It also has, another advantage in that there is no cross-resistance between Colimycin, which will possibly be the trade name of this material, and the other broad-spectrum antibiotics.

DR. KIRBY: How about cross-resistance with polymyxin B?

DR. SCHWARTZ: In general, one does not develop resistance readily to either of these two agents. Those of you who have worked with polymyxin know that it is not easy to develop resistance to polymyxin. This is similarly true of colistymethylsulfate or colistysulfate. Another point is that those organisms which appear resistant in test tube studies are usually true mutants. They do not retain their resistance, and if one stops cultivating these cultures in the presence of the antibiotic, they revert back to sensitive strains.

DR. ROSS: We just had one case which was resistant to both polymyxin and colistin, 0.48 μg/ml, as I recall. This child was a treatment failure and the sensitivity rose from 0.48 to 15.6 μg and at that time the organism was resistant to polymyxin also.

DR. KIRBY: All right, I would like to turn to another subject now. We had some discussion earlier in the week about bactericidal versus bacteriostatic antibiotics and I would like to see if we can apply this specifically to clinical problems. I think that we may make a generalization here that we can get some interesting discussion about. I was very interested in Dr. Romansky's statement that they now think they can cure pneumococcal meningitis with almost any antibiotic if they give large amounts. Originally, of course, the observation was that sulfonamides, extremely bacteriostatic agents, are excellent in treating pneumococcal pneumonia but are a complete failure in treating pneumococcal meningitis because in the spinal fluid you have to kill the pneumococci and there is nothing that kills pneumococci like penicillin. Now Dr. Romansky is telling us that he can treat pneumococcal meningitis with chloramphenicol, tetracycline, or erythromycin. Also, in subacute bacterial endocarditis, there have been many failures with the so-called bacteriostatic antibiotics, chloramphenicol, tetracyclines, and so on. Might this generalization be true, that if one can deliver an adequate amount of any of these antibiotics, the result will be bactericidal? We have done some studies in the last two years in the test tube in bactericidal studies, indicating that this may be true.

In other words, the reason that the broad-spectrum antibiotics are not so bactericidal is that their toxicity precludes delivering enough drug to make them bactericidal. Is it possible that if really much larger doses of these could be given, they would be comparably bactericidal? I personally am inclined to doubt that we can give them in these doses, but I think that Dr. Romansky has stimulated a provocative point of view by saying that they will work in pneumococcal meningitis and I would like to ask him what he thinks of this generalization. Then we want especially to hear from Dr. Sylvester and Dr. Bondi about this.

DR. ROMANSKY: In a sense, this is no different from the problem that arose in the early days of treatment of any form of meningitis with penicillin. You know that in the early 1940's the tremendous dose of 25,000 units of penicillin every three hours was used for pneumococcal and meningicoccal meningitis and the net result was a poor one. It was because of this that penicillin received a poor reputation in terms of treatment of meningitis. And I might point out that penicillin at that time cost about $25.00 for a hundred thousand units, which is, as you can see, why it would be restricted in massive amounts. As soon as it was shown that a million units of penicillin every two hours intramuscularly was satisfactory for meningicoccal meningitis and for pneumococcal meningitis, the trend was reversed. Although there are many people who still use sulfadiazine. I hold against the use of combined therapy when one agent in an adequate dose for an adequate time will do the job. Now as far as the other agents are concerned for the meningitides, I am referring to the chemotherapeutic agents, we have treated severe meningicoccal meningitis with Waterhouse-Frederickson syndrome, shocklike syndromes, with doses of erythromycin, tetracycline and chloramphenicol with results which are satisfactory. Now these satisfactory doses are perhaps two to three times the amount that, for some reason or other, seemed to be the standard dose, whether oral or parenteral. In other words, the tradition seems to be to stick to two grams of this and two grams of that. Well, we use a gram of erythromycin intravenously every four, six or eight hours for these meningitides, or we will use a gram of chloramphenicol in the same fashion, and a half gram or a gram of tetracycline, and I can only say that over a period of ten years the results are satisfactory. There aren't that many cases, frankly, that one can treat for meningicoccal meningitis, but if you noticed in our paper yesterday, over a period of ten years we had 43 patients with pneumococcal meningitis. Quantitatively, it is not a large number, but I personally am not concerned if I cannot use penicillin in the meningitides. I will just use large amounts, four to eight grams a day of the others, intravenously.

DR. KIRBY: Would you do that with endocarditis too?

DR. ROMANSKY: No. Well, let me qualify that, Bill. I have no hesitancy in sensitive green strep endocarditis. I would not, however, do it in enterococcal infections because the literature is just loaded with failures.

DR. KIRBY: Well, it is with the green strep, too, isn't it? The broad-spectrum drugs gave failures in endocarditis, which is why they were abandoned. Is this because the dosage should have been six or eight grams a day? Would this make a difference?

DR. ROMANSKY: Dr. Dowling in 1948, I believe, successfully treated a sizable number of patients with sensitive green strep with aureomycin. How they tolerated it for six weeks I don't know, but he did treat them satisfactorily. And I think, Bill, if enough could be given orally with these other agents, the results in sensitive green strep would be all right.

DR. KIRBY: Now, having heard these clinical comments, I would like to hear what Dr. Sylvester, who has been doing a lot of bactericidal versus bacteriostatic work, has to say about the clinical implications of so-called bacteriostatic antibiotics being bactericidal.

DR. SYLVESTER: This is a subject we could spend a good deal of time on, but I might confine our discussion a little bit to the things that would be pertinent. In this area, we have to specifically designate what we are talking about. Are we talking about bactericidal concentrations or are we talking about bactericidal agents? Because in essence any substance that is biologically active has a concentration at which it is bactericidal. On the other hand, to define what a bactericidal agent is, it is necessary to set up some artificial criteria and it is a purely arbitrary designation.

To get back to the point that I am trying to make, I think that all the antibiotics that we are talking about here do have bactericidal concentrations. The significant point is that the bactericidal concentrations of some of these antibiotics are so high that they are beyond the concentrations that can be effectively reached in the host. From a therapeutic standpoint, then, the question is, with any specific agent, is it possible without getting undesirable side effects, to achieve a concentration of that agent which will have bactericidal action?

DR. KIRBY: Yes. Thank you, Dr. Sylvester. And the thought is that Dr. Romansky has, in pneumococcal meningitis, attained this. Dr. Bondi, would you care to comment?

DR. BONDI: Dr. Kirby, I can't speak with any great authority on this subject, but I would like to add that this is one example of where there is good in vitro and in vivo correlation, in that it is reasonable to suppose, and evidence points this out, that an agent shown to be bactericidal in the test tube is apt to be the agent that will act more rapidly in the body. Not necessarily more effectively, but certainly more rapidly. I know it is a little old-fashioned, and I am talking a little out of order at a meeting like this to speak about immune mechanisms, but at one time we used to make the point that the advantage of a static agent was that it did give the body a little longer period of time to develop immunity that might subsequently be of value in preventing further infection. I wonder what some of our clinicians feel about this?

DR. KIRBY: I want to skip right ahead to another subject where immune mechanisms do come in and that is how do you get rid of boils? This is one thing that many of us get many calls about and say, "Well, I have a patient who has had boils for a year. How do you get rid of them?" And Dr. Bunn has an answer to this, he tells me. To my knowledge there is really no treatment for boils. It doesn't seem to help much to give antibiotics when a patient has a boil and it doesn't seem to help much in eradicating them. All the cleanliness measures have been tried and they don't help very much. Some patients given nasal creams or sprays of bacitracin or neomycin seem to be helped, but when all these things fail, what can you do? What really does get rid of boils? There are even now some papers in the literature that one gives tolbutamide, the antidiabetic agent. Some people think that this chews up the sugar, others feel that tolbutamide has a nonspecific action in the type of dermatitis that often is associated with boils. Are there any other things we can do when all these things fail? Dr. Bunn, how do we get rid of recurrent boils?

DR. BUNN: We undertook a study with these patients for a variety of reasons, primarily, to study host defense mechanisms. We used, by chance, staphylococcal disease because it is a very impressive disease with which measuring rods are fairly simple to observe. Believing there was some difficiency in the mechanisms defending at the skin level, we thought we might be able to supply the patient with some of the deficiencies by giving them gamma globulin in rather large amounts. The gamma globulin which we have been using, and which has been very kindly supplied by the Parke Davis Company, is material which is reasonably high in staphylococcal protective antibodies. And because we were also studying a variety of other defense mechanisms we chose this group of patients. The study is now almost a year old and we have treated very few individuals. Without going into any of the laboratory aspects of it, the clinical impression that we gained from the administration of modest to large amounts of gamma globulin over a period of three to four months has been a delightful experience. I am not making any suggestion, except that as a preliminary observation, the clinical response to the administration to the adult of 10 ml of gamma globulin intramuscularly in one dose, and one week later a second dose, and then monthly thereafter, has been followed after four or five or six weeks with a marked subsidence of the redevelopment of abscesses. I might add that it has absolutely no effect on any immediate disease process, so standard procedures for therapy, such as incision and drainage are just as necessary as ever. But it would appear that we are preventing the recurrence. This is difficult treatment to take but it sounds a little bit sensible to us. We hope eventually to have some laboratory observations which will confirm that we are indeed endowing these individuals with some defense mechanisms manufactured by somebody else.

DR. KIRBY: Tell us what you do again? Now, you inject 10 ml of gamma globulin intramuscularly. You repeat this a week later- and then monthly for six months (for six doses), and then the furuncles disappear. Have we any controls?

DR. BUNN: The patient is actively his own control. In the selection of patients we have started this on, the abscesses have been constantly recurring for a minimum of six months and most of them had been constantly recurring for two years. That was our only control.

DR. KIRBY: Can a patient serve as a control for such a thing, though, because it is characteristic of furuncles that they stop eventually?

DR. BUNN: That is perfectly true, Dr. Kirby, and I would like to make this point right now. We are not recommending this as a proven therapeutic procedure. All I am suggesting is that by pure chance we started our protection studies using this infection as something where we hoped to be able to measure change and that because of the clinical observations we are perfectly willing to suggest that this is a study that probably should be done on a very well controlled basis and in a variety of ways other than in the way we are doing it.

DR. KIRBY: Any comments about this business of using gamma globulin? Dr. Sylvester?

DR. SYLVESTER: I can't comment about this specific thing you just mentioned, but after the gamma globulin discussion I would like to comment on this problem.

DR. KIRBY: All right, let's do a little bit more on the gamma globulin. Dr. Romansky?

DR. ROMANSKY: For some two years now, I have been studying essentially the same problem. Actually we have only had two patients with whom we have had to use gamma globulin, and then we used doses in tremendous quantities, by comparison with Dr. Bunn's, having had some experience with it in other areas, exclusive of staphylococcal infections, in which I know patients can tolerate 50 grams very easily. But the point I would like to make is this. I don't think it has to do so much with what you have used or what is given, but it is how we handle these furuncles, carbuncles, or boils from their very initiation. My experience has been that I too see the patients not very early in the eruption of these boils. If one could get the clinician to apply the basic principles very, very early, in other words, as soon as the first sign of a pimple or furuncle occurred, he would apply heat and simultaneously use antibiotics, I think most of these not only would be aborted rapidly, but the patient would be given a chance to build immunologic protection. I feel very strongly about what Dr. Bondi said. We did not touch upon immunology here and we must remember that the drug, the host, and the parasite are all very important factors here. But if one aborts this infection fast enough, one does give, I believe, the immunological part of the mechanism a chance to build up some immunity. Because this organism is a very invasive one, if we can abort them quickly my experience is that one can gradually spread out the frequency of these recurrences to the point where they will completely disappear, so that we could use this as a control for Dr. Bunn if you want. Not that I am against the gamma globulin, because I think this is a very important part of it. We studied 150 patients with recurrent staphylococcal infections and some 37% of them revealed not a hypo gamma globulinemia, but a hyper gamma globulinemia which I feel personally is just as significant as a hypo gamma globulinemia.

DR. KIRBY: Are you saying, Dr. Romansky, that when a patient gets the first sign of a furuncle, he have a supply of antibiotics at home and immediately take it, apply heat, and try to prevent the infection from ever developing?

DR. ROMANSKY: That is exactly what I have the patients do. They have a supply of what I think is the most effective agent, whether albamycin or erythromycin, or chloromycetin. Tetracycline is the lowest on the totem pole and we don't use it in these cases. The moment a patient who has had these recurrent affairs detects the onset of a furuncle, he immediately applies hot packs for 15-20 minutes three times a day, and attempts to abort the infection in the very early phase. Frankly, this has been a program that has been successful in all but probably two cases, as I recall it.

DR. KIRBY: And during this you don't have a problem of the organisms becoming resistant because you eradicate them.

DR. ROMANSKY: I wouldn't say we eradicate them; that is a broad statement. But what we do find is that over a period of time the incidence of recurrence is spread out and within six to eight weeks (the maximum of about eight weeks) the great majority of them will have occasional little whiteheads occur, then they will disappear altogether.

DR. KIRBY: Now why are you getting better immunization by quickly suppressing the furuncle, instead of by letting it develop?

DR. ROMANSKY: Well, this is speculative. When the infection is present it does not allow the immune system to act the way it normally should with a smaller inoculum. In other words, I think the inoculum is too heavy to allow production of adequate antibodies and I think this is possibly manifest by the hyper gamma globulinemia. We see some aberrant relationship there. Now, this has not been verified.

DR. KIRBY: I think we have two amazingly different regimens here. One hits them hard with antibiotics; the other lets the boils go along, but cures the patient with gamma globulin. I suppose either or both may be witchcraft and probably they feel that way about each other.

DR. SYLVESTER: I have a few comments that might fit into the middle of these two extremes. Some of you will recall the papers that have been published in relation to the potentiation of antibiotics by gamma globulin in experimental animals and also some publications where this has been used in humans. Human studies, obviously, are not too good a control. The animal studies aptly point out that gamma globulin does add to the efficacy of the antibiotic and as such you would assume that it contributes to the host defenses of the infected animal. We also have been studying this question of the immunological characteristics of staphylococci for some years. We feel that we can differentiate to some extent between sera of individuals who are excessively sensitive to staphylococcal infections and those who apparently do not ever have even a superficial staphylococcal infection. We are not quite sure just what we are measuring at the moment, but there is this indication. For example, in sera of cystic fibrosis patients, of chronic osteomyelitis patients, and those with chronic furunculosis, the sera does seem to be lacking in what we would call an antistaph factor. It is not one of the known staphylococcal antibodies.

DR. KIRBY: But is there a factor lacking that you can measure?

DR. SYLVESTER: I am not going to stick my neck out on this because we are not sure yet exactly what we are measuring. We haven't enough data to know whether we are chasing an artifact or not, but we have the impression at this point that we can detect some difference. This is primarily a staphylococcal growth-inhibiting measure, simple inhibition of various dilutions of sera of the growth of the autogenous strain. We also have observed in animals, and this is far from complete, that a certain degree of immunity can be obtained to either a local lesion or to a systemic infection by various types of antigenic preparations of staphylococcal cells. The interesting thing here is that, depending upon how you prepare your antigen, you get a different type of immunity. This we have not completed either. In addition to this, over the past several years we have prepared for some dermatologists in our area, as a service, a number of autogenous vaccines of staphylococci. Their enthusiasm for these is marked. I am not sure that they are really effective, because again there are no controls. But in two instances where we have a fairly decent follow-up over a long period of time, it appears that a long protracted course of immunization with Staphylococcus cells does provide freedom from recurrent furunculosis in an individual who for years previous had had recurrent furunculosis. I think that this is an area where much is still to be learned, but I do feel that it ultimately is going to be possible to develop the right antibodies if we can find the way of preparing the right antigen.

DR. KIRBY: Thank you, Dr. Sylvester. Any general comments or questions about the furuncle situation. Yes, Dr. Rutenberg?

[COMMENTS FROM THE FLOOR: Transcription unintelligible.]

DR. KIRBY: Isn't it true, Dr. Rutenberg, that many surgeons now take the attitude that drainage with no antibiotic is the most appropriate approach to this problem?

A: I think it is rather interesting that where I happpen to be there was a team set up to handle the staphylococcal situation about four years ago and it happens to be essentially the same clinicians who have been seeing all the these patients. The regimen that they started with was almost precisely the same that Dr. Romansky is talking about, emphasizing this idea of heat and the early application of antibiotics. In the last year and a half or so there seemed to be an attempt to modify that slightly and, if anything, they are putting more emphasis on heat and abortion, as Dr. Romansky just said, and possibly following something of a middle ground by taking the relatively few that do not respond to heat alone, and applying antibiotics as quickly and as liberally as they can. Possibly that may be the middle ground and might be more acceptable to the people who don't want to get into antibiotics quite so quickly. It apparently has been very effective on at least 30 of these chronic recurrent cases that have been in our program for a long time.

DR. KIRBY: And what happens? Does the furuncle drain, or does it just go away?

A: I think, from what I see of it, that there appears to be a mixture of responses. Some do just go away. Others go on and head up and under those circumstances they hit them hard and do the surgical drainage as might be indicated.

DR. ROMANSKY: I just want to make one point. The reason that the patients that I have seen have been on an immediate antibiotics and heat regimen is because they have had so many recurrences. I wouldn't necessarily give it to them on the very first furuncle. I wouldn't want that impression to be conveyed.

DR. KIRBY: A question from the audience. When would an autogenous vaccine be used and what have been the results? I think that Dr. Sylvester said that he would certainly like to use the vaccine, but he doesn't know just what the right antigen is. Is that right, Dr. Sylvester?

DR. SYLVESTER: Yes. I certainly would hesitate to subject anyone to a regime or suggest that he be subjected to a regime of vaccination with the current autogenous vaccines because they require anywhere up to three months of continuous administration for any effect at all. If we could get a vaccine that could be administered, say, in two doses, then I think we could readily study this problem.

DR. KIRBY: This is certainly practiced widely, though, and I think there are those who are just as enthusiastic about giving staphylococcal vaccines as Dr. Bunn is about his gamma globulin. Let's see what Dr. Bunn has to say.

DR. BUNN: I would like to make it very clear that I am not enthusiastic about our program at all. You asked me questions and I had to tell you what we did. As a matter for study, I think the use of vaccines are sort of like cold vaccines. Some of them work and some don't. As a matter of fact, most of the patients whom I have seen are the end results of lots of various treatments and practically all of them have been on some kind of a vaccine before I ever see them. I am like Dr. Romansky. I don't see these people on their initial episodes and perhaps we are talking about a different group of patients. I heartily agree with Dr. Sylvester. I think this may be a very real and logical answer for the future, but the materials that are now prepared in most bacteriological laboratories have very little advantage and certain disadvantages. I have yet to see the first good report on a controlled study that would suggest that they do very much good as presently prepared.

A: Three little points. I think the first one is about the autogenous vaccine. We usually use the autogenous vaccine after everything else has been tried and we make it a point, when we have autogenous vaccine being used, that if a patient develops a boil while on this, we add that to the previous one, so we keep adding autogenous vaccine, and I think we are getting better results. For 25 years, they have been using autogenous vaccines in my laboratory and this is the thing that has finally evolved. The second point is, along the lines dealt with by Dr. Romansky, and the use of antibiotics early—we have been using topical antibiotics. You can shove about 30 μg of antibiotic underneath the skin, in the subcutaneous tissues, with topical antibiotic therapy, if you massage the material in. And we have been using this on these people who have multiple furunculosis. We massage in the antibiotic, like bacitracin or erythromycin ointments.

DR. KIRBY: Does this go through the entire skin or just into the draining furuncle?

A: This is over the furuncle when it is starting to develop. In the pimple stage, or earlier preferably, you can abort these. You can see them melt right away, although we don't have controls showing that these would go ahead and develop.

DR. KIRBY: What antibiotic do you rub on the skin?

A: Bacitracin and erythromycin. Then the third point that we have been advocating to these patients, on the basis of the Canadian work that showed the penetration of staphylococci into the mattresses, is to purchase plastic mattress covers and pillow case covers. I have no controls on that.

DR. KIRBY: Thank you, doctor. Well, I think that we have come to the end of our time, and I would like it if we could have some closing remarks from the man who really was very active in lining up this program, Dr. Herman. I would just like to ask Dr. Herman to make any concluding remarks he would like to, including any statements about what might be occurring next year. Dr. Herman.

DR. HERMAN: Thank you, Dr. Kirby. I am most appreciative to you, Dr. Kirby, for the wonderful program you have gotten together this afternoon. For a long time we thought this Conference would have to be called off, but as you can see there hasn't been an empty chair in here since the opening of this panel.

I don't want to keep you any longer, but I do thank one and all of you for having faith in our judgment and for coming here and helping us to produce this first Conference on Antimicrobial Agents. We hope that you got your money's worth, that you enjoyed this program, and that you want us to continue this type of conference! We have been burning the midnight oil for the past three nights, discussing suggestions, getting kicked around, and occasionally losing our tempers, but fortunately we are still all on speaking terms. We have been given the opportunity to reserve these same rooms for a similar conference in this hotel next year for the 15th to the 17th of November. As to the sponsor for the 1961 conference, whether it is to be the SIM, the SAB, or some other organization, I am not certain.

I think we have enough experience so that we should not underestimate the problems. However, we are all working toward a common goal and if everybody pitches in as well as they have this year we will no difficulty. An item on which I hope some one of you can give the committee some concrete ideas is the publication of the book covering these transactions. We will probably break even on this meeting, but the extra cost of publication will require some additional funds. We also need assistance of all kinds, especially for our editor, Dr. Peter Gray, who is going to undertake the editing of all the papers and panels presented at this Conference. He will do a good job and he will have a lot of work, and I know that he will also have a lot of cooperation.

If any of you have any suggestions for a better conference next year, please don't hesitate to send them in to 2000 P Street, N.W., to the Society for Industrial Microbiology Conference on Antimicrobial Agents. Thank you one and all. The 1960 Conference now stands adjourned.

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